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Study of Post-Harvest Physiological Deterioration in Transgenic Cassava

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STUDY OF POST-HARVEST PHYSIOLOGICAL DETERIORATION IN TRANSGENIC CASSAVA

Simon Edward Bull

A thesis submitted for the degree of Doctor of Philosophy

University of Bath

Department of Biology and Biochemistry

July 2011

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ABSTRACT

Cassava (*Manihot esculenta* Crantz) was domesticated approximately 8,000 years ago and is a staple food for over 500 million people in about 105 tropical and subtropical countries. Vegetatively propagated for its starch-rich storage roots, cassava has an exceptional capacity to grow on marginally fertile soils and in regions with low annual rainfall. However, production in Africa - the largest producer of cassava - is constrained by numerous biotic and abiotic factors, including viral infection (e.g. cassava mosaic viruses and cassava brown streak viruses), pests and post-harvest physiological deterioration (PPD). PPD is an endogenous process that renders the roots unmarketable and unpalatable within approximately 24-48 hours after harvest. Although harvesting triggers a wound response, cassava is unable to modulate the accumulation of reactive oxygen species (ROS), resulting in oxidative damage and the development of symptoms referred to as vascular streaking. Over-expression constructs containing selected genes involved in ROS detoxification (*ASCORBATE PEROXIDASE* (*APX*), *CATALASE*, *GALACTURONIC ACID REDUCTASE*, γ -*GLUTAMYL*CYSTEINE SYNTHETASE (*GSH1*) and *SUPEROXIDE DISMUTASE*) and driven by the root-specific *PATATIN* promoter (*StPAT*) were successfully crafted. The protocol for *Agrobacterium*-mediated transformation of friable embryogenic callus (cultivar TMS60444) was extensively modified to guarantee production of transgenic cassava and progress was monitored using constructs harbouring the *GUSPlus* reporter gene. PCR-based analyses and Southern blot hybridisation revealed successful and stable integration of the transgenes with >85% of lines having T-DNA inserted into a single genomic fragment. The *APX* transgene and peroxidase activity were successfully up-regulated in transgenic cassava storage roots. Additionally, enhanced accumulation of the antioxidant thiol, glutathione, was measured in *GSH1* transformed plants. Unique data elucidating suitable reference genes to study transgene expression profiles using real-time PCR is provided. And experiments to develop an assay to measure PPD in glasshouse-cultivated storage roots were performed. The data presented in this thesis aims to expand our knowledge of cassava tissue culture, transformation, PPD and prolong the shelf-life of cassava storage roots *via* enhancement of ROS-detoxifying pathways.

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The research transcended two time zones, which I think explains why I frequently took one step forward and two steps back. To my friends and colleagues at ETH Zürich (Switzerland) I thank Christophe Laloi (now hiding in the south of France) for his endless patience despite me haranguing him with questions about qPCR and, far more importantly, for being a reliable source of some exceptional wines. To Willi Gruissem for inspirational enthusiasm whilst in his group and Hervé Vanderschuren for his dedication, moral support and helpful discussions throughout the project. To Simona Eicke, Judith Owiti, Isabel Moreno, Evans Nyaboga, Simona Pedrussio, Gaëlle Messerli, Kim Schlegal, Oliver Kötting and a special thanks to Samuel Zeeman for all their kindness and friendship whilst in Zürich.

Finally, I leave you with the thought that the vast majority of my project was spent troubleshooting, optimising protocols and trying to understand new techniques. During this time I was frequently awash with anecdotal information and often reminded of a line in the film *Where Eagles Dare*....."right now I'm about as confused as I ever hope to be!"

ABBREVIATIONS

$^1\text{O}_2$	singlet oxygen
ACMV	African cassava mosaic virus
APX	ascorbate peroxidase
BAP	6-benzylaminopurine
bp	base pair
BSA	bovine serum albumin
CAM	cassava axillary medium
CAT	catalase
CBM	cassava basic medium
CBSD	cassava brown streak disease
cDNA	complementary DNA
CEM	cassava elongation medium
CIAP	calf intestinal alkaline phosphatase
CIAT	international centre of tropical agriculture
CIM	cassava induction medium
CMD	cassava mosaic disease
CMM	cassava maturation medium
COM	cassava shoot organogenesis medium
C_T	threshold cycle
DIG	digoxigenin
ds	double stranded
DTT	dithiothreitol
<i>E</i>	amplification efficiency
EST	expressed sequence tag
FEC	friable embryogenic callus
FW	fresh weight
FzW	frozen weight
g	gram
GalUR	galacturonic acid reductase
GD	Gresshof & Doy medium
GFP	green fluorescent protein
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidised glutathione
GST	glutathione transferase
GUS/GUSPlus	β -glucuronidase
H_2O_2	hydrogen peroxide
HCN	hydrogen cyanide
HO^\bullet	hydroxyl radical
HPLC	high performance liquid chromatography
HPRG	hydroxyproline-rich glycoprotein
hr	hour
HPX	horseradish peroxidase
IITA	international institute of tropical agriculture
Kb	kilobase
KJ	kilojoule
L	litre
LB	Luria-Bertani medium
M	molar
MB	monobromobimane

mg	milligram
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
MS	Murashige & Skoog medium
MV	methyl viologen
mwt	molecular weight
N	normal
NAA	1-naphthaleneacetic acid
NEFC	non-embryogenic friable callus
NFW	sterile, nuclease free water
ng	nanogram
nm	nanometer
O ₂ ^{•-}	superoxide anion radical
OD	optical density
PAL	phenylalanine ammonia lyase
PCD	programmed cell death
PCR	polymerase chain reaction
POX	peroxidase
PPD	post-harvest physiological deterioration
PSI & II	photosystem I & II
rfA	reading frame cassette A
RFP	red fluorescent protein
ROS	reactive oxygen species
rpm	revolutions per minute
RT	reverse transcription
S.D.	standard deviation
S.E.	standard error
SDW	sterile, distilled water
SH	Schenk & Hildebrandt medium
SOD	superoxide dismutase
SOSG	singlet oxygen sensor green
StPAT	<i>PATATIN</i> promoter
T _m	melting temperature
U	unit
UV	ultraviolet
v/v	volume/volume
w/v	weight/volume
x g	gravitational force
γ-EC	γ-glutamylcysteine
γ-GCS	γ-glutamylcysteine synthetase
μg	microgram
μl	microlitre
μm	micrometre
μM	micromolar

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1 INTRODUCTION

1.1 CASSAVA: A WORLD CROP

1.1.1 History and phylogeny

Cassava is an ancient crop that was domesticated approximately 8,000 years ago. Its origin is a debated topic and based on recent phylogenetic analyses Léotard *et al.* (2009) propound it to be in the south western Amazonian rim, whilst Duputié *et al.* (2011) suggest it was in Mesoamerica (south west Mexico; Figure 1.1). During the 16th Century cassava was transported by Portuguese sailors to west Africa and originally grown only in the Gulf of Guinea. However, an increase in trade led to cultivation of the crop in central regions of Africa and by the 18th Century it was farmed in the provinces of East Africa, where plants were probably introduced from Madagascar and *via* Indian Ocean trade routes. Cultivation expanded rapidly and by the 20th Century cassava was grown throughout all sub-Saharan Africa and South and South East Asia.

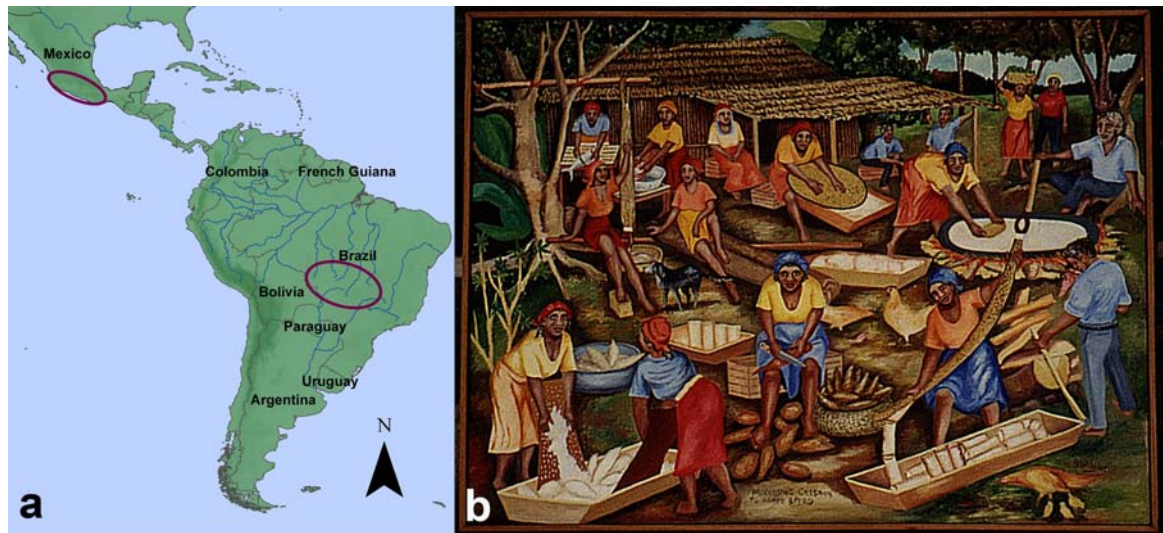


Figure 1.1 Origin and domestication of cassava. Proposed origins (circled) of cassava (a); map generated using ArcGIS (Version 9). Processing of cassava in South America (b; *source of image unknown*).

Cassava (genus *Manihot*) belongs to the family Euphorbiaceae, which also includes agriculturally and economically important crops such as rubber (*Hevea brasiliensis*), castor bean (*Ricinus communis*) and jatropha (*Jatropha curcas*; Abdulla *et al.*, 2011). Between 11 and 19 groups of *Manihot* have been described based on plant morphology and eco-geographic similarities, comprising trees (group Glaziovianae), perennial sub-shrubs (Tripartitae and Graciles) to nearly acaulescent sub-shrubs (group Stipularis; Allem, 2002; Pax, 1910; Rogers & Appan, 1973). Within these groups approximately 98 species of *Manihot* have been catalogued that are all monoecious except for those in the group Stipularis that are dioecious. Female (staminate) flowers open 1-2 weeks before the male (pistillate) flowers and are normally cross-pollinated by insects, resulting in a highly heterozygous gene pool. The domesticated crop (*M. esculenta* Crantz; Figure 1.2a) - a shrub that typically grows 1-4 m in height - is also known as manioc, yuca and tapioca and is closely related to two sub species *M. esculenta* ssp. *Flabellifolia* and *M. esculenta* ssp. *Peruviana* that are regarded as the wild progenitors (Allem, 2002).



Figure 1.2 Cassava (*M. esculenta* Crantz). Plant grown in India (photograph by S. E. Bull) (a) and harvested storage roots in Kenya (photograph courtesy of Charles Orek) (b).

1.1.2 Cassava storage root anatomy

Cassava is grown primarily for its starch-rich storage roots (Figure 1.2b) that are differentiated from adventitious roots. Consequently these roots lack the meristematic tissue/bud primordia present in true, stem tissue-derived tubers such as potato (*Solanum tuberosum*) that facilitate dormancy and reproduction (Morris & Taylor, 2010). The mature cassava storage root comprises several tissue layers that can be grouped into three categories, (i) the bark or periderm, (ii) the peel, including the bark, cortical parenchyma and phloem, (iii) edible parenchyma, comprising cambium, storage parenchyma and xylem vessels (Figure 1.3; Cabral *et al.*, 2000; Hunt *et al.*, 1977). The peel accounts for approximately 11-20% of the root weight and is removed prior to processing (Montagnac *et al.*, 2009a). The anatomy of cassava roots is studied rarely, but a recent investigation into cellular organisation and structure of 1-3 month old developing adventitious roots in wild (*M. glaziovii* and *M. fortalezensis*) and domesticated (*M. esculenta* cultivar UnB 122 and UnB 201) varieties revealed greater numbers of xylem vessels in the domesticated cassava and also variation in the lignification of cell walls. These observations likely reflect the hybrid origin of cassava and the crops tolerance to drought and disease (Bomfim *et al.*, 2011).

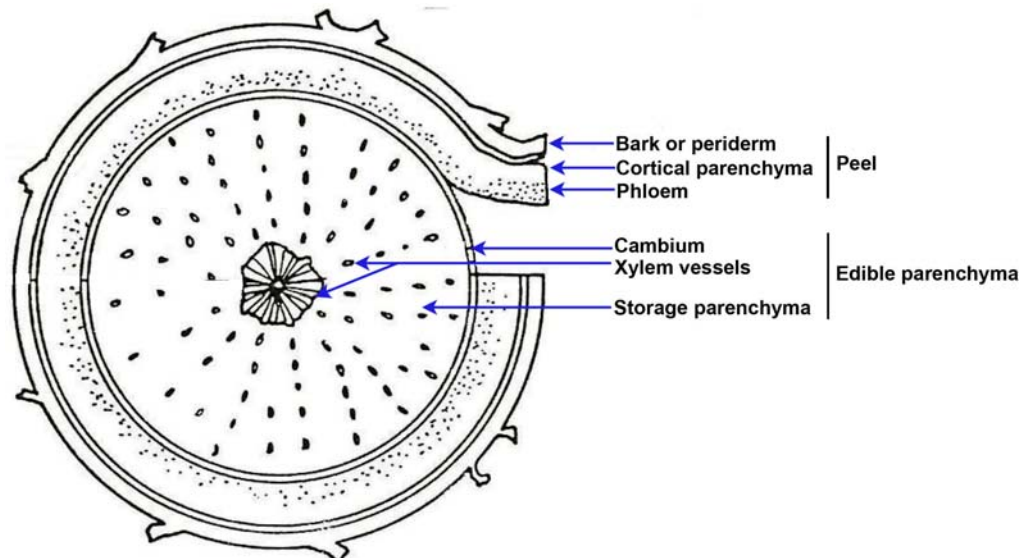


Figure 1.3 Cassava storage root anatomy. Diagram modified from Hunt *et al.* (1977).

1.1.3 Importance, uses and cultivation of cassava

Cassava is a staple food providing as much as a third of daily calorie intake for approximately 500 million people in about 105 countries (FAO, 2008). Starch accounts for approximately 80% of the root dry weight ensuring that cassava yields more energy per hectare (1045 KJ hectare⁻¹) than other major crops, such as rice (652 KJ hectare⁻¹; Montagnac *et al.*, 2009a). Thus, in the developing world cassava is amongst the top four most important crops (with rice, sugarcane and maize) and global production in 2009 is estimated at 233 million tonnes (FAOSTAT, 2009a). Africa, where cassava is grown primarily for food, is the largest producer with yields estimated to exceed 118 million tonnes per year (Figure 1.4; FAOSTAT, 2009a). Cassava as a food is prepared in a variety of ways that differ between continents and countries. Boiling, mashing, frying and drying are widely used to produce granules, flour and chips that have a seemingly endless list of applications. In west Africa cassava is often processed into *gari* – the cassava is pulped, fermented for 3-10 days and then heated to form a semolina. A typical Brazilian product is *polvilhoazedo* (fermented starch used in baking), whereas in Cameroon the resplendently named *Meduame-M-Bong* (boiled and washed roots) is prepared and eaten with meats and fish (Balagopalan, 2002). In Asia and South East Asia the crop is grown mainly for animal feed and industrial purposes. For example, sweeteners, acids, alcohols, biodegradable plastics and there is also growing interest in using cassava as a source of biofuel (Balat & Balat, 2009; Jansson *et al.*, 2009).

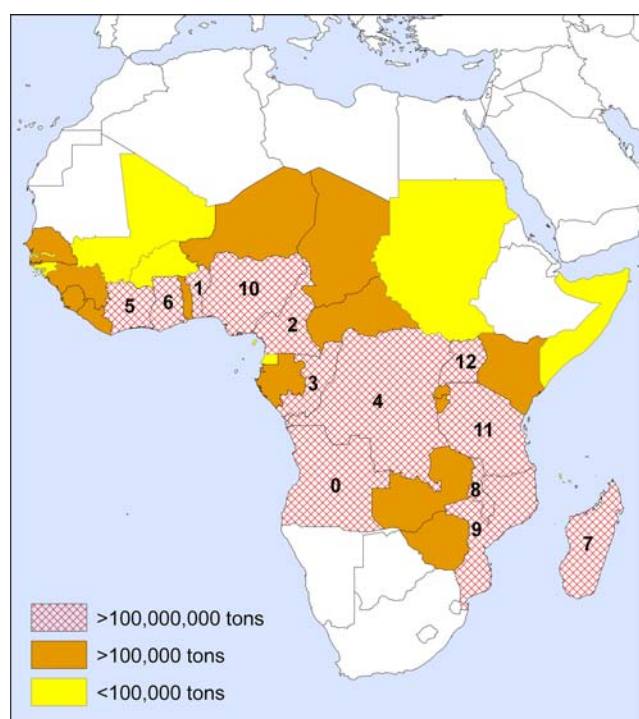


Figure 1.4 Cassava production in Africa. Country labels: Angola (0), Benin (1), Cameroon (2), Congo (3), Democratic Republic of Congo (4), Côte d'Ivoire (5), Ghana (6), Madagascar (7), Malawi (8), Mozambique (9), Nigeria (10), Tanzania (11) and Uganda (12). Data gathered from FAOSTAT (2009a) and collated using ArcGIS (Version 9).

Cassava is vegetatively propagated *via* stem cuttings that are used to multiply stocks and for planting. Approximately five to ten cuttings, which are typically 20 cm in length, can be obtained from a single plant. This approach ensures that farmers are not required to purchase seed or are reliant upon seed generation, which seldom occurs in *M. esculenta*, probably as a consequence of extensive domestication. Furthermore, in times of famine the farmer does not consume the “seed” of cassava, unlike other staple crops such as maize. Cassava is frequently intercropped with other staple foods (e.g. maize) and is grown in regions 30°N to 30°S in a range of agro-ecologies, including marginally fertile soils, variable rain-fed conditions (from 600 mm per year in semi-arid tropics to 1000 mm in humid tropics) and at temperatures between 25-35°C (El-Sharkawy, 2004). The tolerance of cassava to drought and other environmental stresses means that when other crops fail cassava roots can usually still be harvested (Burns *et al.*, 2010). However, despite these advantageous traits cassava production is generally mediocre with current yields barely averaging 20% of those obtained under optimal conditions, particularly in Africa (Fermont *et al.*, 2009).

1.2 PROBLEMS ASSOCIATED WITH THE CONSUMPTION AND PRODUCTION OF CASSAVA

1.2.1 Nutrient content and cyanogenic glucosides

Cassava is rich in carbohydrates but the roots have very low quantities of minerals, protein and vitamins compared with the leaves (Table 1.1; Montagnac *et al.*, 2009a). Cassava also contains large amounts of cyanogenic compounds that are converted to hydrogen cyanide (HCN) following tissue disruption and catalysis by enzymes (e.g. β -glucosidases; Blagbrough *et al.*, 2010; Burns *et al.*, 2010; Zagrobelny *et al.*, 2008). A bitterness in taste caused by these compounds usually deters insects and herbivores. Although there is wide variation in the concentration of cyanogenic compounds between cultivars, non-bitter roots generally have <100 mg HCN equivalents kg⁻¹ fresh weight (FW), whereas bitter roots may contain >450 mg HCN equivalents kg⁻¹ FW (Chiwona-Karlun *et al.*, 2004; Sundaresan *et al.*, 1987). Various processing techniques listed above can remove more than 96% of the cyanogens and thus reduce cassava toxicity for consumption (Montagnac *et al.*, 2009b). Occasionally, however, consumption of the bitter varieties - usually at times of drought and/or war - can cause serious illness, especially in children who may experience stunted growth and irreversible paralysis of the legs (Nhissco *et al.*, 2008; Nzwalo & Cliff, 2011).

Table 1.1 Nutrient content of cassava roots and leaves.

Nutrient*	Roots	Leaves
Vitamin A (µg)	5 - 35	8300 - 11800
Vitamin C (mg)	14.9 - 50	60 - 370
Protein (g)	0.3 - 3.5	1 - 10
Carbohydrate (total, g)	25.3 - 35.7	7 - 18.3
Zinc (ppm)	14	71

* Approximate quantity of selected nutrients, vitamins and mineral per 100 g tissue. Data collated from Montagnac *et al.* (2009a).

1.2.2 Biotic and abiotic stresses

Cassava production in Africa is greatly constrained by several biotic factors, including cassava green mite (Skovgård *et al.*, 1993), cassava mealy bug, cassava bacterial blight (Boher & Verdier, 1994), cassava brown streak disease (CBSD; Hillocks & Jennings, 2003) and cassava mosaic disease (CMD; Patil & Fauquet, 2009). CMD is caused by whitefly-transmitted begomoviruses (family *Geminiviridae*) for which several species have been identified throughout cassava growing regions of Africa (Berrie *et al.*, 2001; Bull *et al.*, 2006; Bull *et al.*, 2003; Hong *et al.*, 1993; Stanley & Gay, 1983). The disease - characterised by a yellow-green mosaic of the leaves, leaf distortion, stunted growth and decrease in the size of root - is probably the most significant biotic constraint to cassava production in Africa. Although the true incidence and severity of CMD is difficult to quantify (Sseruwagi *et al.*, 2004), *African cassava mosaic virus* (ACMV) alone is estimated to cause 28-40% crop losses totalling 28-49 million tonnes per year (Thresh *et al.*, 1994; Thresh *et al.*, 1997). CBSD is also the result of a viral infection (cassava brown streak viruses) and characterised by brown streaking symptoms in the storage root. There is only scant information about CBSD compared to CMD, especially concerning virus transmission, but recent publications offer new insights into the molecular characteristics of the virus and disease aetiology (Mbanzibwa *et al.*, 2011; Winter *et al.*, 2010), providing new tools and knowledge to evolve disease resistance programmes.

Cassava production is also hindered by numerous abiotic factors that include infertile soils, post-harvest root deterioration, planting of unimproved traditional varieties and inadequate farming practices. The planting of sub-optimal material, for example

unimproved varieties or diseased cuttings, is exacerbated by the fact that cassava is vegetatively propagated. Without an organised and systematic dissemination of disease-free and improved cultivars, inferior material may be grown and distributed between farmers. This problem is often compounded by inefficient planting densities, as well as poor weed, pest and disease management (Hillocks, 2002). Unfortunately, even effective farming practices and good yields can be significantly impeded by post-harvest physiological deterioration (PPD). The storage root functions as an energy reserve to the plant and thus there is no selective advantage to repair wounds and damage to the root following harvest. Ordinarily the root deteriorates within 1-2 days after harvest, which in village societies is generally not a major problem since roots are harvested and consumed when required. However, with an increase in cassava production for marketing and industrial processes, PPD significantly affects crop losses, root quality, economic costs, marketability, consumer availability and commercial processes (Page & Beeching, 2011). For example, starch extraction rates are reported to be significantly reduced in processing plants in Latin America and Indonesia. Additionally, in Thailand - the largest exporter of cassava-based products - the crop is grown close to processing plants to minimise deterioration and freshly harvested roots are used daily. As a consequence of PPD, some urban consumers and processors import other sources of carbohydrate, exacerbating the problems for rural farmers (Onwueme, 2002; Plumbly & Rickard, 1991).

1.3 POST-HARVEST PHYSIOLOGICAL DETERIORATION

1.3.1 Biochemical and molecular understanding

PPD was first reported in Argentina in 1928 and described by Castagnino (1943) as the appearance of blue/black veins, a symptom later referred to as 'vascular streaking' (Averre, 1967; Figure 1.5). This phenotype develops within 48 hours after harvest (Drummond, 1953) and arises in the xylem parenchyma at wound sites and later in storage parenchyma (Booth, 1976; Montaldo, 1973). Early research revealed microorganisms are not involved in PPD since none could be cultured from freshly deteriorated areas of the root and treatment with fungicides and bactericides failed to prevent PPD, indicating vascular streaking is an endogenous process (Noon & Booth, 1977). It was later concluded that the blue/black product is due to the oxidation of hydroxycoumarins by peroxidases and hydrogen peroxide (H_2O_2). Hydroxycoumarins are secondary metabolites that are involved in plant defence and include esculetin and scopoletin. Application of phenolic compounds to freshly harvested root sections

revealed that only scopoletin caused a rapid and intense discolouration indicative of PPD (Wheatley & Schwabe, 1985). The synthesis of scopoletin *via* the phenylpropanoid pathway in cultivar MCOL22 increases during PPD, peaking 24 hours after harvest at 100 nmol g⁻¹ FW as measured using High Performance Liquid Chromatography (HPLC), before gradually returning to basal levels (approximately 20 nmol g⁻¹ FW) in subsequent days (Buschmann *et al.*, 2000b). Accumulation can also be visualised since hydroxycoumarins fluoresce under ultraviolet (UV) light (Buschmann *et al.*, 2000b; Wheatley & Schwabe, 1985). Interestingly, there was no correlation between quantification of fluorescence and subjective scoring of symptoms in 25 cultivars of cassava roots after five days storage. This discrepancy was attributed to stabilisation and gradual degradation of hydroxycoumarin content prior to symptom development (Salcedo *et al.*, 2010). The involvement of scopoletin in defence and PPD was implicated further since phenylalanine ammonia lyase (PAL), a key enzyme in its production, was up-regulated following treatment of cassava cell suspension cultures with pathogens including *Fusarium oxysporum* (Gómez-Vásquez *et al.*, 2004) and increased levels of the protein have also been detected within 24 hours post harvest (Owiti *et al.*, 2011). Similarly, a 17% increase in PAL activity has been reported in sweet potato following wounding and storage for two days at 15°C (Reyes *et al.*, 2007). The biosynthetic pathway for scopoletin in harvested cassava roots is being elucidated using HPLC and mass spectrophotometer techniques (Bayoumi *et al.*, 2008a; 2010; Bayoumi *et al.*, 2008b) and which may offer insights into the factors affecting its production and regulation.

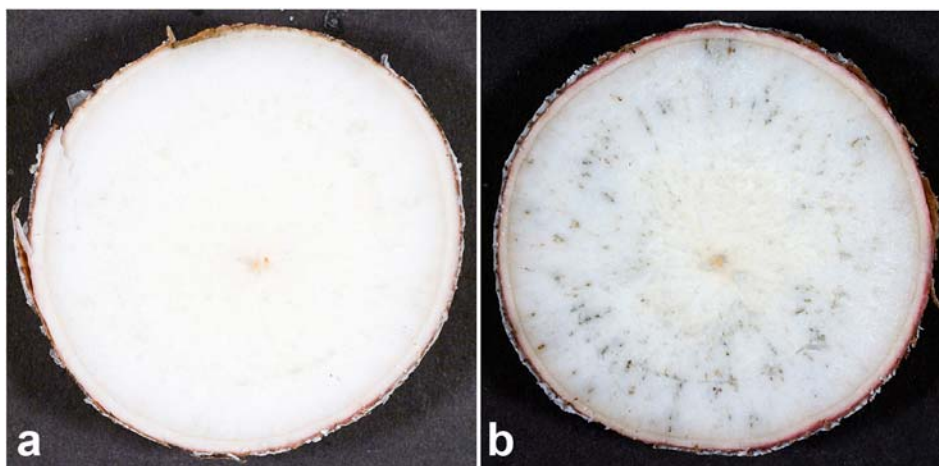


Figure 1.5 Cross-sections of harvested cassava root. Symptomless root immediately following harvest (**a**) and PPD symptoms (vascular streaking) 48 hour post-harvest (**b**). Unknown cultivar acquired from supermarket (UK). Photographs by J. Beeching (University of Bath).

PPD resembles a wound response that manifests changes in cell wall structure, lipid composition (Lalaguna & Agundo, 1989), increased ethylene synthesis and respiration, programmed cell death and a wound-induced oxidative burst (Beeching *et al.*, 1998; Reilly *et al.*, 2004). An increase in the phytohormone ethylene has been detected in cassava approximately 16 hours post-harvest (Hirose *et al.*, 1984), although during late PPD (>48 hour post-harvest) protein accumulation of 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (the enzyme involved in the rate limiting step in ethylene production) is down-regulated (Owiti *et al.*, 2011). In other root crops, such as potato tubers and sugarbeet, wounding also led to an increase in ethylene biosynthesis. Interestingly, in potatoes this did not appear to be associated with wound healing (suberisation; Lulai & Suttle, 2004) and in sugarbeet an increase in respiration was also detected but which did not detrimentally affect storage (Fugate *et al.*, 2010). Thus whilst an increase in ethylene biosynthesis is commensurate with a wound response, its role during PPD remains unclear. An increase in respiration required to provide energy for defence pathways has also been detected within 24 hours of cassava root harvest (Hirose *et al.*, 1984) and increased approximately 186% in wounded sugarbeet four days after harvest (Lafta & Fugate, 2011). Fluctuations in antioxidant capacity (measured *via* changes in, for example, phenolic compounds, ascorbate, glutathione and carotenoids) have also been catalogued in harvested/wounded root crops. Wegener & Jansen (2010) reported significant increases in ascorbate and phenolic compounds in potato. Whilst Reyes *et al.* (2007) concluded that potato and sweet potato tissue with high levels of ascorbate are intrinsically better prepared for wounding. As such, synthesis of phenolic compounds are directed for lignin production and suberisation. The formation of protective barriers *via* the accumulation of lignin, suberisation and crosslinking of hydroxyproline-rich glycoproteins (HRGPs; reviewed by Deepak *et al.*, 2010) is an important aspect of the plant wound response and which has been observed in cassava (Han *et al.*, 2001; Owiti *et al.*, 2011; Reilly *et al.*, 2007). Lastly, programmed cell death (PCD) – a controlled process for cellular suicide - has also been implicated in plant wound defence (Gadjev *et al.*, 2008). It is not known whether cellular breakdown during PPD is PCD or simply physical disruption, but studies are currently underway to understand better the process in transgenic cassava (K. Jones, *pers. comm.*). However, an increase in cysteine proteases (caspases) that are involved in cleaving proteins and thus instigating cell death, have been detected in protein analyses during PPD, especially 48-72 hour post harvest (Owiti *et al.*, 2011) and also in a microarray (Reilly *et al.*, 2007), suggesting that PCD is to some extent implicated in PPD. The complex interrelation between the

numerous pathways associated with a wound response ensures difficulty in unravelling the biochemical and molecular processes during PPD. However, despite this, recent publications using advanced techniques such as peptide tagging and microarray analysis (cited above) are providing researchers with an overview of PPD and the opportunity to highlight potentially important pathways and products. Amongst these, the production and control of reactive oxygen species has been pinpointed and forms the basis of research presented in this thesis.

1.3.2 Reactive oxygen species and their involvement in PPD

Reactive oxygen species (ROS) and their detoxification have been implicated in PPD. ROS are molecules that are derived from non-toxic molecular oxygen (O_2) and include singlet oxygen (1O_2), superoxide anion radical ($O_2^{\bullet-}$), H_2O_2 and hydroxyl radical (HO^{\bullet}). In plants, ROS are produced during the normal metabolism of photosynthesis and respiration, involving photosystems I and II (PSI and PSII), mitochondrial electron transport chain, membranes and peroxisomes. The photosynthetic centre PSII generates 1O_2 due to insufficient energy dissipation through carbon fixation, instead transferring excitation energy from chlorophyll to O_2 . 1O_2 can be physically quenched by compounds such as carotenoids that deactivate 1O_2 to O_2 (Triantaphylidès & Havaux, 2009). 1O_2 oxidises amino acids and causes membrane damage and is unique amongst ROS since $O_2^{\bullet-}$, H_2O_2 and HO^{\bullet} are generated *via* a series of reduction reactions. $O_2^{\bullet-}$ can be formed from PSI, PSII and membrane NADPH oxidases and has a short life span (Møller *et al.*, 2007). Unable to cross membranes, $O_2^{\bullet-}$ can be dismutated by superoxide dismutase that exist in different isoforms and results in the production of H_2O_2 (discussed in Chapter 7). Although less reactive than $O_2^{\bullet-}$, H_2O_2 readily permeates membranes and is therefore capable of disrupting enzymes *via* oxidation of their thiol groups. H_2O_2 is removed by catalases (located in glyoxysomes and peroxisomes) and peroxidases (POX) located in different cellular compartments, but particularly chloroplasts. The final reductive stage (Fenton reaction) gives rise to HO^{\bullet} that has extremely high oxidising potential compared to the other ROS and cause significant cellular damage (Garg & Manchanda, 2009). Importantly, although ROS are toxic by-products of aerobic reactions, $O_2^{\bullet-}$ and H_2O_2 serve crucial roles in signalling and defence gene activation (Galvez-Valdivieso & Mullineaux, 2010; Møller & Sweetlove, 2010; Triantaphylidès & Havaux, 2009). Under stress conditions, such as high light intensity, drought and wounding (Jaspers & Kangasjarvi, 2010), an increase in ROS production is therefore co-ordinately balanced

between accumulation, scavenging and signalling to prevent sustained oxidative damage.

An oxidative burst occurs within 15 minutes of cassava root harvesting and is hypothesised to be the trigger for PPD (Reilly *et al.*, 2004). H₂O₂ production was detected following vacuum infiltration of root sections with 3,3-diaminobenzidine tetrahydrochloride (DAB), revealing accumulation in the cortical parenchyma within 24 hours after harvest and later in the storage parenchyma (Buschmann *et al.*, 2000a). A rapid H₂O₂ burst (approximately 2-3 minutes) has also been detected in cassava cell suspension cultures exposed to pathogens including species of *Fusarium* (Gómez-Vásquez *et al.*, 2004). More sophisticated analysis of ROS production and modulation is starting to emerge providing detailed insights into their involvement in PPD. The presence of ¹O₂ has been identified at parenchyma cell walls and close to the site of wounding using a singlet oxygen sensor green (SOSG) probe, appearing within only four hours after harvest (Iyer *et al.*, 2010). A microarray analysis of roots undergoing PPD revealed that many of the 63 up-regulated (≥1.8 fold) genes had roles in ROS generation and modulation, including catalase (EC 1.11.1.6; Reilly *et al.*, 2001), ascorbate peroxidases and secretory peroxidases (EC 1.11.1.7), all of which are involved in H₂O₂ detoxification (Reilly *et al.*, 2007). Some of these findings are supported by recent iTRAQ-based analysis of cassava root proteome with increases detected for superoxide dismutase during early (6-24 hours) and for catalase during late (48-96 hours) PPD (Owiti *et al.*, 2011). Importantly, ROS detoxification not only relies upon enzymatic reactions but also the involvement of antioxidant compounds such as glutathione (Foyer & Noctor, 2011; Mahmood *et al.*, 2010; Chapter 6). Although harvesting triggers a burst of ROS that cause a cascade of defence responses, it appears that in cassava a typical wound response is inadequate. In particular, wound repair appears to be lacking in cassava (Beeching *et al.*, 1998; Han *et al.*, 2001) allowing a continued imbalance between stress and homeostasis.

1.4 DETERIORATION IN OTHER TROPICAL TUBER CROPS

1.4.1 Sweet potato (*Ipomea batatas*; Family Convulaceae)

Cassava is particularly susceptible to PPD but all tuberous crops, including sweet potato, yam and cocoyam are classed as perishable (compared to grain crops; Page & Beeching, 2011). In 2009, sweet potato was the 13th most important crop in the world

with the vast majority (>80%) of production in China (FAOSTAT, 2009b). Sweet potato is usually propagated from vine cuttings that give rise to tuberous roots which function as propagules. The tubers contain 50-80% (dry weight) starch and are a source of vitamin C and provitamin A. However, like cassava they have a poor protein content (estimated 5%) comprising predominately the storage protein sporamin (Shewry, 2003). The tubers adaptation for dormancy (albeit relatively brief) offers some explanation as to why the crop can be stored for weeks or months depending on the cultivar and storage conditions (Onwueme, 1978). The construction of thatched covered pits is a common practice in almost all sweet potato growing countries and where roots can be stored for approximately eight weeks (Gooding & Campbell, 1964). However, yields are afflicted by post-harvest losses as a consequence of physical damage, microbial infection (e.g. *Fusarium* rot) and due to pests such as sweet potato weevils (*Cylas formicarius*). The relatively thin and delicate skin of roots is easily scrapped or bruised during harvest, with 25% of crops being damaged even prior to transport to market – a process that further exacerbates the propensity for crop losses (Ray & Ravi, 2005).

In sweet potato, respiration, sprouting and biochemical fluctuations indicative of a wound response contribute to weight loss and unfavourable root characteristics. Respiration peaks within 24 hours after harvest but gradually decreases, as does starch content, during storage (Picha, 1986). Interestingly, the rate of respiration increased in high O₂ concentration environments, suggesting an involvement of ROS possibly as signalling molecules (Chang & Kays, 1981). Sprouting is also a major problem since it occurs rapidly in sweet potato, especially when stored at high temperature and humidity. However, storing roots in structures that provide a temperate climate (i.e. 14°C and diffused light) can suppress sprouting by 99% (Data, 1988). Similar to cassava, harvesting and subsequent spoilage due to pathogens induces a wound response with heightened expression of genes in the phenylpropanoid pathway, including *PAL* whose expression peaks within 24 hours and is accompanied by increases in POX activity and phenolic compound accumulation. Interestingly, however, vascular streaking and extensive oxidative damage ostensibly does not arise in sweet potato. The reason for this is unknown but may be associated with the rapid curing and suberisation of exposed parenchyma cells to form a wound periderm, thus reducing O₂ flux and continued post-harvest damage. Optimal conditions for curing are reportedly 29-33°C and 80-95% humidity for 4-7 days prior to storage at approximately 14°C and 90% humidity (Picha, 1986; Ray & Ravi, 2005). Importantly, the expansion of conventional breeding

programmes and advances in biotechnology (Yang *et al.*, 2011) should provide new options to combat post-harvest losses in sweet potato.

1.4.2 Yams (*Dioscorea* spp.; Family Dioscoreaceae) and cocoyams (Family Araceae)

Yams (e.g. *Dioscorea rotundata* Poir; white yam) are grown predominantly in west Africa with Nigeria ranked as the largest producer worldwide (Arnau *et al.*, 2010; FAOSTAT, 2009c). The dioecious plant is relatively tolerant of dry conditions but growth is severely restricted at temperatures below 20°C and require fertile soils to grow well. Marginal soils that can support cassava or sweet potato are unlikely to be adequate for yam production; soil in the yam growing regions of west Africa is, generally, relatively high in phosphorous (Onwueme, 1978). Yams can be propagated by vine cuttings, seed or tuber, although seed production is highly variable and on average only 5-6 seeds may be obtained from a single female plant of *D. rotundata*. Propagation by tuber is by far the most common and it is the attributes of the tuberous root that, of the root species discussed here, ensures yams are probably the least susceptible to deterioration. Derived from the hypocotyl (region of stem between the radicle and cotyledons), yam tubers comprise meristematic tissue serving as propagules and, unlike sweet potato, have a tough cork periderm providing a protective barrier to damage, pathogens and water loss (Arnau *et al.*, 2010).

Post-harvest storage of yams affects various parameters, including sugar and phenolic content and respiration. In *D. alata* ("Florido") and *D. cayenensis-rotundata* ("Krenglè"), phenolic compounds were in greater abundance in proximal root tissue compared to distal parts, although content throughout the tuber decreased during storage (0-6 months). Conversely, sugar content increased during storage and was most abundant in distal tissue, probably due to starch hydrolysis (Kouakou *et al.*, 2010). Suppression of respiration and water loss *via* effective wound healing/lignification following harvest is similar to sweet potato and dependent upon optimal light, humidity and temperature (Passam *et al.*, 1977; Passam & Noon, 1977). Various techniques are applied to provide the optimal balance and 'yam barns' are a common sight in west Africa (Onwueme, 1978). However, achieving optimal storage conditions in tropical and sub-tropical developing countries can be challenging. Moreover, given the tuberous characteristics of yam, a more arresting problem is the prevention of sprouting. Recent studies utilising *in vitro* grown microtubers have assessed various environmental conditions upon

dormancy, concluding that reduced temperature (i.e. approximately 18°C) rather than light is more important to minimise tuber sprouting (Ovono *et al.*, 2010). Like cassava and sweet potato, yams are also susceptible to microbial infection, in particular to fungal species *Fusarium* and *Aspergillus*, especially following wounding. Indeed, pathogen infection is considered the most significant cause of post-harvest losses in yam cultivation (Aboagye-Nuamah *et al.*, 2005).

The cocoyams including tannia “new cocoyam” (*Xanthosoma sagittifolium*) and taro “old cocoyam” (*Colocasia esculenta*) are edible aroids that require average daily temperatures above 21°C and a plentiful water supply. The corms and cormels are rich in starch and, in general, post-harvest losses are largely due to microbial infection following wounding (Onwueme, 1978). As for sweet potato and yam, storage conditions affect post-harvest losses due to increased rate of respiration leading to weight loss and the conversion of starch to sugars. Factors that not only influence storage but also crop characteristics as a food source. Under tropical ambient conditions reduced weight loss, respiration rates and decay were attributed to effective curing of wounds - a process that is promoted by high temperatures (i.e. >20°C) - although the impact upon tissue varies between different species. However, under conditions of low temperature (15°C) and high humidity (85%) cormels of both tannia and taro could be stored for approximately 5-6 weeks (Agbor-Egbe & Rickard, 1991). These various studies for sweet potato, yam and cocoyam underline the intrinsic differences between cassava storage roots and other tropical root crops. Of particular note is the inability of cassava roots to serve as propagules, lack of dormancy and the incapacity to establish an effective wound periderm following harvest/damage, resulting in continuous accumulation of ROS and stress induced defence responses.

1.5 TECHNIQUES TO DELAY PPD IN CASSAVA ROOTS

1.5.1 Traditional approaches

There are numerous traditional approaches to minimise PPD in cassava, including pre-harvest pruning and various storage techniques. Pruning the foliage of MCOL22 plants 2-3 weeks prior to root harvest resulted in only 4% of roots being deteriorated after 20 days in storage; in comparison, approximately 96% of roots were deteriorated from unpruned plants (Rickard & Coursey, 1981). The effects of pre-harvest pruning have also been assessed in six cultivars with varying susceptibility to PPD; MCOL22 and SM627-5

are highly susceptible, MCOL72 and MVEN77 moderately susceptible, whilst MBRA337 and MPER245 are least susceptible. Following pruning at intervals 0-39 days before harvest, susceptibility to PPD in all cultivars was reduced overall to less than 25% of unpruned plants in roots stored for 25 days following harvest. However, whilst pruning may prolong the shelf-life of cassava roots, the procedure affects root qualities due to an increase in sugar content, presumably as a result of starch hydrolysis (van Oirschot *et al.*, 2000). Another approach commonly used on small farms is simply to retain roots in the ground until they are required. However, the plants are therefore more susceptible to pests and diseases and the roots become increasingly woody. Significantly, it also means the valuable land is being utilised simply as a means of storage when it could be used for new harvests and other crops – certainly not a feasible option for cassava grown for commercial processes (Westby, 2002). Other techniques include coating the roots in wax and wrapping in air-tight bags to exclude oxygen (Wheatley & Schwabe, 1985). However, these techniques are time-consuming and expensive for such a low cost commodity and suitable only for export to markets that are prepared to pay a high price for cassava.

1.5.2 Conventional breeding and biotechnology

Traditional farming techniques discussed above have been complemented with continuous advances in both knowledge and technology aimed at improving cassava. Conventional breeding programmes have long been key in encouraging these advances and resulted in the introgression of important traits into the cassava germplasm with improvements recorded for bacterial blight resistance, virus resistance (Hahn *et al.*, 1980; Okogbenin *et al.*, 2007), protein content (Chávez *et al.*, 2005), starch quality (Ceballos *et al.*, 2007) and PPD (Morante *et al.*, 2010), as well as in developing techniques such as marker-assisted breeding (Rudi *et al.*, 2010). Marker-assisted breeding is estimated to reduce by several years the cycle for conventional breeding and developing resistance to PPD alone has been predicted to save \$3 billion over a 25 year period in sub-Saharan African countries (Rudi *et al.*, 2010). However, traditional breeding remains fraught with limitations, notably the heterozygous nature of the crop renders it difficult to identify the true breeding value of parental lines, poor fertility and introgression of the selected trait(s) into farmer-preferred cultivars without affecting their favoured characteristics remains difficult (Ceballos *et al.*, 2004; Kawano, 2003; Nassar & Ortiz, 2010). Thus, production of improved plant lines by conventional breeding can take approximately 10 years from the first parental crossing to distribution of the improved

plants (M. Fregene, *pers. comm.*). Notwithstanding these complications, several cultivars have been developed recently that are remarkably resistant to PPD. Of particular note are GM905-66, AM206-5 and WAXY4 that were totally devoid of PPD symptoms even after 40 days storage (Morante *et al.*, 2010). The basis for PPD resistance in these clones has not been conclusively defined but for GM905-66 it is likely to be associated to high carotenoid content (Sánchez *et al.*, 2006). AM206-5 and WAXY4 are amylose-starch mutants (Ceballos *et al.*, 2007) and it has been suggested that the waxy-starch gene may be linked to PPD, although the precise relation is unclear (Morante *et al.*, 2010). Despite the constraints in breeding programmes, the progressive development of cultivars with improved nutritional and agronomic traits collectively broadens our knowledge of factors affecting PPD and thus provide possible targets for its control (Chávez *et al.*, 2005).

Biotechnology both complements and facilitates breeding programmes and an Expert Consultation by the FAO viewed biotechnology as the most appropriate technique to resolve PPD in cassava (Wenham, 1995). To date, however, biotechnology has not been used directly to combat PPD and itself remains a problematic field of research. The lack of progress in generating transgenic plants has been attributed to numerous difficulties, including financial burdens, a need for appropriate facilities, lengthy process and an apparent lack of common knowledge and skills. These problems and constraints are addressed in detail in Chapter 4. The development of transgenic plants is a key aspect for researchers involved in the BioCassava Plus programme, who seek to improve zinc, iron, protein (Abhary *et al.*, 2011) and vitamin A content, reduce levels of cyanogenic compounds, develop disease resistance and extend the shelf-life of cassava roots (Blagbrough *et al.*, 2010; Sayre *et al.*, 2011). Advances in molecular mapping (Akano *et al.*, 2002; Okogbenin *et al.*, 2007), sequencing of cDNA clones and expressed sequence tags (ESTs; Lokko *et al.*, 2007; Sakurai *et al.*, 2007) and specifically the recent elucidation of the cassava genome sequence (Cassava Genome Project 2009), all provide tools for these biotechnology-based projects. Furthermore, recent advances in proteome technology also provide detailed information regarding gene expression profiles during PPD (Owiti *et al.*, 2011) and embryogenesis (Baba *et al.*, 2008). An internationally promoted goal is to transfer the skills and knowledge surrounding biotechnology to laboratories in developing countries, in particular Africa, to ensure the necessary infrastructure is in the hands of those who seek to gain from the exciting new advances in cassava biotechnology (Bull *et al.*, 2011).

1.6 RESEARCH OBJECTIVES

Research into cassava PPD has in recent years revealed the significant involvement of enzymes and antioxidant compounds in the production and detoxification of ROS. The ultimate aim is to prolong the shelf-life of cassava storage roots *via* the generation of transgenic plants over-expressing selected genes driven by a root-specific promoter. The specific research objectives were:

- (a) Adapt a binary expression cassette (pCAMBIA 1305.1) to allow efficient cloning of selected genes: *ASCORBATE PEROXIDASE* (*MecAPX2*), *CATALASE* (*MecCAT1*) and *SUPEROXIDE DISMUTASE* (*MecSOD2*) isolated from cassava. *GALACTURONIC ACID REDUCTASE* (*GalUR*) from strawberry and γ -*GLUTAMYL CYSTEINE SYNTHETASE* (*GSH1*) isolated from *Arabidopsis*. *MecAPX2*, *MecCAT1* and *MecSOD2* encode enzymes involved in detoxification of hydrogen peroxide and superoxide radicals. *GalUR* and *GSH1* are involved in the production of the antioxidant compounds ascorbate and glutathione, respectively. The genes will be driven by a root-specific promoter (*StPAT*) from *PATATIN*, which encodes the major storage protein in potato.
- (b) Isolate the regulatory sequence of cassava *MecPX3*, which encodes a putative secretory peroxidase. Gene expression is root specific and up-regulated during PPD, suggesting it may be an ideal promoter for future studies.
- (c) Critically appraise the cassava transformation protocol using the model cultivar TMS60444. The ability to generate transgenic plants is of paramount importance for success of this project.
- (d) Generate *in vitro* transgenic cassava plantlets using the created expression constructs. These plantlets will be characterised to identify independent lines and confirm integration of the transgene.
- (e) Establish an infrastructure for growing cassava plants for storage root production in a glasshouse environment at the University of Bath. Roots will be assessed for PPD and preliminary molecular and biochemical analyses will be performed to characterise the selected transgenic plants.

2 MATERIALS & METHODS

2.1 DNA AMPLIFICATION FOR CLONING AND ANALYSIS

2.1.1 Polymerase chain reaction (PCR) for target sequence amplification

PCR incorporating proof-reading polymerase was used to isolate the coding regions of genes involved in modulation of ROS as described in Chapter 3. Reactions were prepared in sterile 0.2 ml thin-walled PCR tubes and consisted of approximately 100 ng DNA template, 5 μ l 10X KOD DNA polymerase buffer, 2 μ l of 25 mM $MgCl_2$, 5 μ l of 2 mM dNTPs, 1.5 μ l of 10 μ M forward primer (Table 2.1), 1.5 μ l of 10 μ M reverse primer (Table 2.1), 1 μ l KOD DNA polymerase (Novagen) and sterile, distilled water (SDW) to 50 μ l. Reactions were cycled in a PTC-200 Peltier Thermal Cycler (MJ Research) at 94°C (3 min) and then 25 cycles of 94°C (40 sec), 50-60°C* (40 sec), 72°C for a time dependent upon expected amplicon length (1 min per 1 Kb amplification), and a final step of 72°C for 10 min. * The annealing temperature was adjusted to approximately 5°C below the melting temperature (T_m) of the primers and within the range of 50-60°C. Amplification products were visualised by agarose gel electrophoresis (Section 2.4.2).

2.1.2 PCR amplification for genotyping/screening

PCR using *Taq* DNA polymerase was used to check successful ligation and cloning of DNA fragments, as well as for screening transformed bacteria and plant material. 20 μ l reactions comprised 2 μ l template DNA or lysate (Sections 2.2.8 and 2.3.1), 2 μ l of 10X ThermoPol buffer (New England Biolabs; NEB), 4 μ l of dNTPs (1.25 mM), 1 μ l of 10 μ M forward primer (Table 2.1), 1 μ l of 10 μ M reverse primer (Table 2.1), 0.2 μ l of *Taq* DNA polymerase (NEB) and 9.8 μ l of SDW. Reactions were cycled in a PTC-200 Peltier Thermal Cycler (MJ Research) at 94°C (3 min), followed by 25 cycles of 94°C (40 s), 50-60°C* (40 s) and 72°C for a time dependent upon expected amplicon length (1 min per 1 Kb amplification), and a final step of 72°C for 10 min. * The annealing temperature was adjusted to approximately 5°C below the melting temperature (T_m) of the primers and within the range of 50-60°C. Amplification products were visualised by agarose gel electrophoresis (Section 2.4.2).

2.1.3 Quantitative real-time PCR and data analysis

Real-time PCR was used to determine expression levels of transgenes and reference genes in cassava plants. 1 μ l of cDNA (Section 2.6.4), 0.5 μ l of 10 μ M forward primer

(Table 2.1), 0.5 µl of 10 µM reverse primer (Table 2.1), 10.5 µl molecular grade, nuclease-free water (NFW; Sigma-Aldrich) and 12.5 µl SYBR® *Premix Ex Taq*™ (TaKaRa) was combined in sterile 0.2 ml tubes maintained on ice before transferring 20 µl of the mix to LightCycler® capillaries (Roche). To minimise pipetting errors, master mixes were prepared where possible. Capillaries were capped, centrifuged at 400 x g (pulse setting) and loaded into the carousel of the LightCycler® (Roche) real-time PCR machine. Data was collected using the LightCycler® software (Version 1.5). Duplicates of each sample were prepared and C_T values used for comparative expression analysis using the formula $2^{-\Delta CT}$ (Livak & Schmittgen, 2001):

$$2^{-CT(\text{Transgene}) - CT(\text{Reference gene})}$$

PCR amplification efficiencies were calculated ($E = 10^{(-1/\text{slope})}$) using the slope of a standard curve generated from a dilution series of cDNA as template DNA. Efficiencies are represented as a percentage ($\%E = (E-1) \times 100$). An optimal slope is -3.32, which translates into $E=2$ and refers to a doubling in the amount of DNA per cycle. Comparative primer efficiencies were determined between a selected reference gene and target gene using the $2^{-\Delta CT}$ formula and the standard deviation (S.D.) calculated: $S.D. = \sqrt{S.D._1^2 + S.D._2^2}$

2.2 CLONING & BACTERIAL TRANSFORMATION TECHNIQUES

2.2.1 TA cloning

The desired fragments derived from PCR amplification (Section 2.1.1) were cloned into the TA vector (pCR®2.1-TOPO®) as directed by the manufacturer (Invitrogen). PCR by KOD DNA polymerase (Section 2.1.1) generates blunt-ended fragments due to the proof-reading capability of the enzyme and thus deoxyadenosine overhangs were added in a separate step to enable cloning. 1-7 µl of PCR product was combined with 1 µl of 10X ThermoPol buffer, 1 µl of 2 mM dATP, 1 µl of *Taq* DNA polymerase (NEB) and SDW to 10 µl in sterile 0.2 ml thin-walled PCR tubes. The reaction was incubated at 70°C for 20 min in a PTC-200 Peltier Thermal Cycler (MJ Research). 1 µl of the reaction mix was removed for TA cloning and used to transform One Shot® TOP10 Chemically Competent *E. coli*, as outlined by the manufacturer (Invitrogen).

2.2.2 Gateway® cloning of target sequence

The Gateway® cloning system was used to transfer each of the target coding regions into the expression vector *via* an intermediate vector; the strategy is discussed in detail in Chapter 3. The PCR products including the added terminal *attB* sites were cloned into pDONR™/Zeo (Invitrogen) and used to transform One Shot® Omnimax 2-T1 Chemically Competent *E. coli* in accordance with the manufacturer's guidelines. The following reaction used the LR Clonase™ II Enzyme Mix (Invitrogen) to transfer the target sequence into the expression cassette for transformation of One Shot® *ccdB* Survival™ T1R Chemically Competent *E. coli* (Invitrogen).

2.2.3 Conversion to a Gateway® compatible system

The Gateway® Vector Conversion System was used to convert pCAMBIA 1305.1 into a Gateway® compatible vector using Reading Frame A (rfA), according to the manufacturer's guidelines (Invitrogen). Further details are provided in Chapter 3.

2.2.4 Preparation of electrocompetent *Agrobacterium tumefaciens* LBA4404

Agrobacterium tumefaciens LBA4404 (henceforth referred to simply as *Agrobacterium*) was used in the transformation of cassava. 10 ml of YEP broth (1% peptone, 1% yeast extract, 0.5% NaCl, 0.5% sucrose, pH 7.5) containing 50 µg ml⁻¹ rifampicin and a colony of *Agrobacterium* was cultured for approximately 48 hr at 28°C, shaking 200 rpm. 5 ml was used to inoculate 500 ml of YEP and cultured at 28°C, 200 rpm until the optical density (OD)₆₀₀ = 0.5-1, as determined by a spectrophotometer (GeneQuant, Pharmacia Biotech). The culture was retained on ice and centrifuged at 2790 x g at 4°C for 15 min. The pellet was resuspended in 500 ml of ice cold 1 mM HEPES/KOH buffer (pH 7.0) and centrifuge at 2790 x g for 15 min at 4°C. Cells were resuspended in 250 ml of ice cold 1 mM HEPES/KOH buffer (pH 7.0) and centrifuged as previously. The cells were resuspended in 200 ml 10% (v/v) glycerol at 4°C, centrifuged as previously and then resuspended in 1.5 ml 10% (v/v) glycerol at 4°C. 40 µl aliquots were transferred to sterile 0.5 ml microfuge tubes, flash frozen in liquid nitrogen and stored at -70°C.

2.2.5 Electroporation of *Agrobacterium* LBA4404

Agrobacterium were transformed with plasmid DNA (expression vectors) *via* electroporation. Approximately 2 µl of plasmid DNA (Sections 2.2.6 and 2.2.7) was added to 50 µl of *Agrobacterium* (Section 2.2.4) and immediately transferred to an ice-cold electroporation cuvette (Bio-Rad, 2 mm gap). Electroporation was performed as a

single pulse at 2.5 kV using a MicroPulser (Bio-Rad). 750 µl sterile Luria-Bertani (LB) media (25 g Luria broth (Sigma-Aldrich) in 1 L SDW) was immediately added to the mix and then incubated at 28°C with shaking (200 rpm) for 2 hr. The culture was spread onto LB agar plates (40 g Luria agar (Sigma-Aldrich) in 1 L SDW) supplemented with 50 µg ml⁻¹ rifampicin, 50 µg ml⁻¹ kanamycin, 100 µg ml⁻¹ streptomycin and incubated for 40 hr at 28°C.

2.2.6 Small scale preparation of plasmid DNA (Minipreps)

Bacterial colonies were selected from LB agar culture plates using a sterile inoculation loop and cultured in 5 ml LB media containing the appropriate antibiotic(s). *E. coli* cultures were incubated overnight at 37°C with shaking (200 rpm), whilst *Agrobacterium* cultures were incubated for approximately 40 hr. DNA was extracted using the QIAprep[®] Spin Miniprep Kit (Qiagen) and eluted in 50 µl SDW.

2.2.7 Midi scale preparation of plasmid DNA (Midipreps)

100 ml LB media containing the appropriate antibiotic(s) was inoculated with a colony of *E. coli* or *Agrobacterium* from a LB agar culture plate. Liquid cultures were shaken (200 rpm) at 37°C overnight for *E. coli* and approximately 40 hr for *Agrobacterium* cultures. DNA was extracted using the QIAGEN Plasmid Midi Kit (Qiagen) and eluted in 50 µl SDW.

2.2.8 Preparation of bacterial colonies for PCR screening/genotyping

Bacterial colonies were pre-treated to disrupt cell structures and improve amplification efficiency for PCR genotyping/screening (Section 2.1.2). Reactions comprised 25 µl of T0.1E buffer (10 mM Tris-HCl (pH8), 0.1 mM EDTA), 1 µl of 0.7 mg ml⁻¹ proteinase K (Sigma-Aldrich) and a bacterial colony selected using a sterile inoculation loop. Reactions were prepared in sterile 0.2 ml thin-walled PCR tubes and cycled in a PTC-200 Peltier Thermal Cycler (MJ Research) at 55°C (15 min) then 80°C (15 min). Subsequently, 2 µl of lysate was used in PCR amplification (Section 2.1.2).

2.3 ISOLATION & CLONING OF CASSAVA GENOMIC DNA

2.3.1 Isolation of genomic DNA from *in vitro* material

Three small/medium sized leaves from an *in vitro* plantlet were transferred to a sterile 1.5 ml microfuge tube containing approximately 200 µl of sterile glass beads (1 mm

diameter). The tubes were immersed in liquid nitrogen before homogenising the tissue to a fine powder using an amalgamator (Silamat® S5) for 6 sec. 1 ml of extraction buffer (50 mM Tris-HCl (pH8), 100 µg proteinase K (Sigma-Aldrich), 2% (v/v) SDS, 100 mM LiCl, 10 mM EDTA (pH8)) was added to the sample and incubated at room temperature on a shaker (100 rpm) for 15 min. The samples were centrifuged at 16,100 x g at 4°C for 15 min. Approximately 700 µl of the supernatant was transferred to a sterile 2 ml microfuge tube and 5 µl of 20 mg ml⁻¹ RNase A (Invitrogen) was added and samples incubated at 37°C for 1 hr. Following incubation, 1 ml of phenol (pH 8.0; Sigma-Aldrich) was added, the tube shaken vigorously and then centrifuged at 16,100 x g for 5 min at room temperature. The upper phase was transferred to a sterile 2 ml microfuge tube containing 1 ml phenol:chloroform (1:1), the sample vigorously shaken and centrifuged at 16,100 x g for 5 min at room temperature. The upper phase was again transferred to a sterile 2 ml microfuge tube containing 1 ml of phenol:chloroform:isoamylalcohol (25:24:1). The sample was vigorously shaken and centrifuged at 16,100 x g for 5 min at room temperature. This step was repeated and the supernatant was then mixed with 0.25X volume of 10 M ammonium acetate in a sterile 1.5 ml microfuge tube. 2.5X volume of cold (-20°C) absolute ethanol was added to the sample, tube inverted several times and the samples incubated at -20°C for 30 min to aid DNA precipitation. Following incubation, the samples were centrifuged at 16,100 x g at 4°C for 25 min. The supernatant was discarded and the pellet resuspended in 750 µl SDW. 750 µl of phenol:chloroform:isoamylalcohol (25:24:1) was added and the sample mixed gently and then centrifuged at 16,100 x g for 5 min. The aqueous phase was transferred to a sterile 1.5 ml microfuge tube containing 0.25X volume of 10 M ammonium acetate and 2.5X volume cold (-20°C) absolute ethanol was added. The tube was gently inverted to mix and incubated at room temperature for 5 min to aid precipitation. Samples were centrifuged at 16,100 x g at 4°C for 15 min and the supernatant discarded. The pellet was washed in 1 ml 70% (v/v) ethanol by inverting the tube several times and centrifuged at 16,100 x g for 10 min at room temperature. The pellet was air dried and resuspended in 100 µl SDW. Samples were stored at -20°C.

2.3.2 Preparation of plating cells for lambda phage

50 ml LB media containing 0.2% (w/v) maltose and 10 mM MgSO₄ was inoculated with a colony of *E. coli* XL1-Blue MRA (P2) (Stratagene) and incubated at 37°C, shaking (200 rpm). The culture was grown to an OD₆₀₀ = 1 and centrifuged at 1780 x g for 10 min. The supernatant was collected and diluted to an OD₆₀₀ = 0.5 using ice cold 10 mM MgSO₄.

2.3.3 Infection of plating cells with phage

Lambda particles in SM buffer (100 mM NaCl, 8 mM MgSO₄, 50 mM Tris-HCl (pH7.5), 0.002% (w/v) gelatin) were added to 200 µl of plating cells (Section 2.3.2) in a 15 ml falcon tube and incubated at 37°C for 20 min. 4 ml of top agar (LB agar, 0.8% (w/v) agarose) maintained at 45°C was added and the tube was inverted to mix. The sample was poured on pre-warmed (37°C) LB agar plates supplemented with 0.2% (w/v) maltose and 10 mM MgSO₄. Plates were incubated overnight at 37°C. Various dilutions of particles were plated.

2.3.4 Purification of lambda phage DNA

Lambda DNA was isolated from plaques extracted using a sterile pipette tip and cultured in 24.5 ml of LB media (supplemented with 0.5 ml 20% (w/v) maltose, 25 µl 1 M MgCl₂, 25 µl 1 M CaCl₂) and shaken (200 rpm) overnight at 37°C. 100 µl of chloroform was added and shaken for 1 min. Culture was centrifuge at 16,100 x g for 10 min and 2 µl of 10 mg ml⁻¹ DNase I and 20 µl of 10 mg ml⁻¹ RNase were added to the collected supernatant. Samples were incubated at 37°C for 45 min. 17 ml of extraction buffer (20% (w/v) PEG (8,000 mwt), 2 M NaCl, 10 mM Tris-HCl (pH8), 10 mM MgCl₂) was added and cooled on ice for 2-3 hr. Samples were centrifuged at 16,100 x g for 10 min at 4°C and the pellet resuspended in 500 µl TE buffer (10 mM Tris-HCl (pH8), 1mM EDTA) and transferred to a 1.5 ml microfuge tube. 5 µl of 10% (w/v) SDS and 10 µl 5 M NaCl were added and the mix shaken. An equal volume (approximately 500 µl) of phenol:chloroform (1:1) was added and shaken vigorously for 15 min before centrifuging at 16,100 x g for 10 min. The aqueous layer was transferred to a sterile 1.5 ml microfuge tube and an equal volume (approximately 500 µl) of chloroform was added, shaken vigorously for 15 min and centrifuged at 16,100 x g for 10 min. The supernatant was added to an equal volume of absolute isopropanol (approximately 500 µl), mixed gently and retained at -70°C for 30 min. The sample was centrifuged at 16,100 x g at 4°C for 10 min and the pellet washed with 1 ml 70% (v/v) ethanol and centrifuged at 16,100 x g for 10 min (room temperature). The pellet was air dried and resuspended gently in 100 µl of SDW.

2.3.5 GenomeWalker™ Universal Kit

The GenomeWalker™ Universal Kit was used as directed by the manufacturer (Clontech Laboratories) using genomic DNA extracted from cassava cultivar TMS60444 (Section 2.3.1). The kit generates pools (or “libraries”) of adaptor-ligated genomic DNA fragments following digestion by restriction enzymes *DraI*, *EcoRV*, *PvuII* and *StuI*. Amplification

primers specific to the adaptors (AP1 and AP2; Table 2.1) and known target sequence (Table 2.1; Chapter 7) were used in nested PCR using the Advantage[®] 2 PCR Enzyme System (Clontech Laboratories; Figure 2.1). Fragments were cloned (Section 2.2.1) and the nucleotide sequence elucidated (Section 2.4.9).

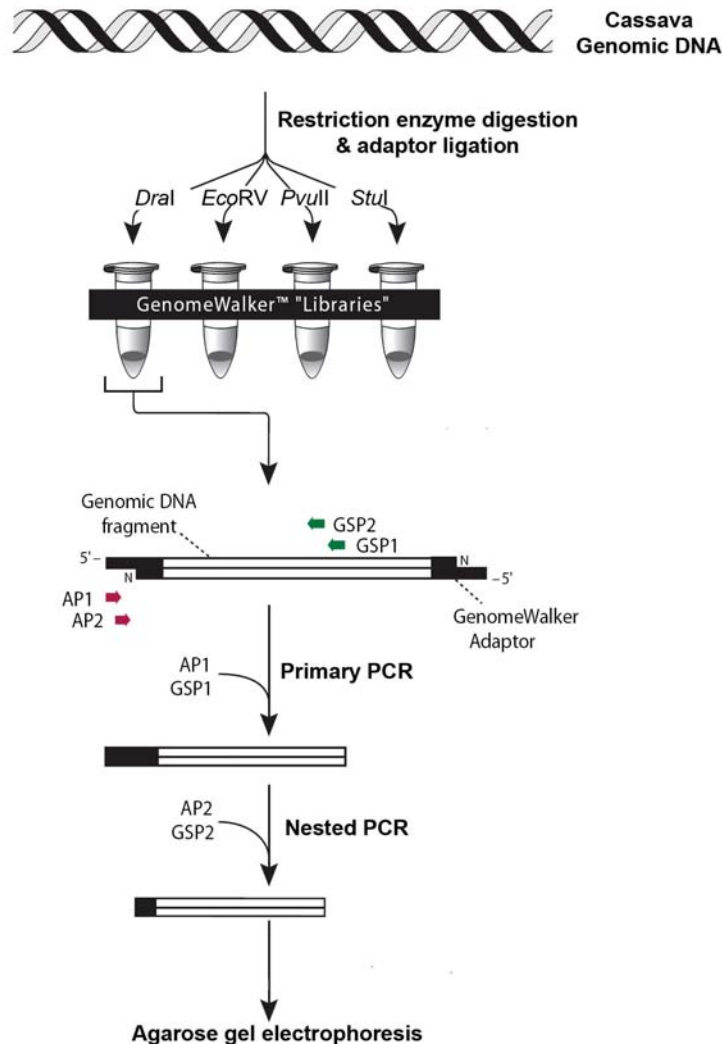


Figure 2.1 GenomeWalker™ protocol. Samples of cassava genomic DNA are digested using restriction enzymes *DraI*, *EcoRV*, *PvuII* and *StuI* and the fragments ligated to GenomeWalker™ adaptors. N: amine group in adaptor to prevent 3' extension. Adaptor specific primer (AP1) and a gene specific primer (GSP1) are used to amplify fragments from the libraries. A subsequent, nested PCR using AP2 and GSP2 primers ensure specific amplification of target sequence that can be observed in ethidium bromide-stained agarose gels. Figure modified from GenomeWalker™ Universal Kit User Manual (Clontech Laboratories).

Table 2.1 Oligonucleotides used to PCR-amplify target sequence.

Primer name	Nucleotide sequence (5' → 3')*
apxF1	AAA GCA GGC TCA AAA ATG CCG AAG AAC TAC C
apxR1	AAG CTG GGT GTT ACG CCT CAG CAA ATC C
antiapxF1	AAA GCA GGC TCC TTA CGC CTC AGC AAA TCC
antiapxR1	AGA AAG CTG GGT GAT GCC GAA GAA CTA CC
catF1	AAA AGC AGG CTC GGT CAT GGA TCC TTG CAA G
catR1	GAA AGC TGG GTC TCA TAT ATT TGG CCT CAC G
anticatF1	AAA GCA GGC TCG TCA TAT ATT TGG CCT CAC
anticatR1	GAA AGC TGG GTA ATG GAT CCT TGC AAG TTC
gshF2	AAG CAG GCT CTA CCA TGG CGC TCT TGT CTC
gshR2	GAA AGC TGG GTG TTA GTA CAG CAG CTC TTC
antigshF1	AAA GCA GGC TCC TTA GTA CAG CAG CTC TTC G
antigshR1	GAA AGC TGG GTT ATG GCG CTC TTG TCT CA
garF1	AAA AAG CAG GCT ACA CCA TGG CAA AGG TTC C
garR1	GAA AGC TGG GTC TCA TAA TTC TTC GTC AAC TTC C
antigarF1	AAA GCA GGC TCG TCA TAA TTC TTC GTC AAC
antigarR1	GAA AGC TGG GTC ATG GCA AAG GTT CCT TC
sodF1	AAA GCA GGC TCG ACA ATG GTG AAG GCC GTT G
sodR1	AGA AAG CTG GGT CCT ATC CTT GCA AAC CA
antisodF1	AAA GCA GGC TAC CTA TCC TTG CAA ACC A
antisodR1	GAA AGC TGG GTT ATG GTG AAG GCC GTT GC
Pat-Pst F	CCT CTG CAG TTG TAG TTA ATG CGT ATT AG
Pat-Pml R	CCT CGT CAC GTG CAT ATA ACA AGC ATG G
GUSattfor	AAA AGC AGG CTC GAT GGT AGA TCT GAG GGT A
GUSattrev	GAA AGC TGG GTG TCA CAC GTG ATG GTG ATG G
attB1	GGG GAC AAG TTT GTA CAA AAA AGC AGG CT
attB2	GGG GAC CAC TTT GTA CAA GAA AGC TGG GT
M13F	GTA AAA CGA CGG CCA G
M13R	CAG GAA ACA GCT ATG AC
DESTSeq F1	CAT CAC TAA TGA CAG TTG CGG TGC
DESTSeqR1	GCA CAT ACA AAT GGA CGA ACG G
CAMBSeqF1	GGC TCG TAT GTT GTG TGG AAT TG
CAMBSeqR1	GCT ATG TAA TAT TTA CAC CAT AAC C
Hygro-For	CCA CTA TCG GCG AGT ACT TCT ACA CAG C
Hygro-Rev	GCC TGA ACT CAC CGC GAC GTC TGT C
HygII F	TCT CGA TGA GCT GAT GCT TTG G
HygII R	AGT ACT TCT ACA CAG CCA TCG G
PP2A-LP2	TGC AAG GCT CAC ACT TTC ATC

PP2A-RP2	CTG AGC GTA AAG CAG GGA AG
18S For	ATG ATA ACT CGA CGG ATC GC
18S Rev	CTT GGA TGT GGT AGC CGT T
SNARE For	GAA GAG GTT TCT GAA GGA TCT CG
SNARE Rev	CCT ACC CAT CTG AGT ATT GTC CCA
UBC-F	ATT CAG GCA ATC TTC TAC GA
UBC-R	GCT CCA CAC TCA TTC ACA AC
Transgene R	GTC ACC AAT TCA CAC ATC ACC AC
APX Transgene-F	TAT GCT GCT GAT GAA GAG GC
UBQ10-F	TGC ATC TCG TTC TCC GAT TG
UBQ10-R	GCA AAG ATC AAT CGT TGT TGA
GCS Transgene F	GGT CAG AAC AGG AGT TAC GCC
AP1	GTAATACGACTCACTATAGGGC
AP2	ACTATAGGGCACGCGTGGT
PX3-GSP1	GACAAAGCAGTCATGGGCGAAGAGGCGAAG
PX3-GSP2	GAGCAGAAGAAGTAGGAAGAAGGAGAGAG
PX3-GSP3	ATGGAAAGCAAAATGAGCTTCCTGGTTC
PX3-GSP9	GATACCGAGTACAATCTGTTCTGA

* The partial and full-length *attB* recombination site sequences (blue font), start codons (yellow highlight), stop codons (grey highlight), restriction sites (red font) and Kozak sequences (italicised font). Oligonucleotides synthesised by Sigma-Aldrich (UK) or Microsynth AG (Switzerland).

2.4 DNA MANIPULATION & CHARACTERISATION

2.4.1 Purification of PCR products

PCR samples were purified using the QIAquick PCR Purification Kit (Qiagen) and eluted in 20 μ l SDW, according to the manufacturer's guidelines. Samples were quantified as outlined in Section 2.4.8.

2.4.2 Agarose gel electrophoresis

DNA samples were mixed with blue/orange 6X loading buffer (Promega) and electrophoresed in an ethidium bromide-containing (0.25 μ g μ l⁻¹) 1% (w/v) agarose gel. Gels were prepared and electrophoresed in 1X TBE buffer (89 mM Tris-borate, 2 mM EDTA (pH8)) at 100V. For large DNA fragments (e.g. lambda and genomic DNA) 0.8% (w/v) gels were prepared with TAE buffer (40 mM Tris-acetate, 1 mM EDTA (pH8)) and electrophoresed overnight at 10V. The DNA was viewed using a GDS 7500 UV

transilluminator (UVP) and Grab-IT 2.0 software. Samples were co-electrophoresed with Quick-load[®] 1 Kb Ladder (NEB), Lambda DNA *Hind*III Digest (NEB) or Quick-load[®] 100 bp DNA Ladder (NEB) depending on expected fragment size.

2.4.3 DNA isolation from agarose gels

The QIAEX[®] II Gel Extraction Kit (Qiagen) was used according to the manufacturer's guidelines to isolate desired fragments from agarose gels.

2.4.4 Restriction enzyme digestion of DNA

Digestion of plasmid DNA, PCR products and genomic DNA was achieved using restriction enzymes (NEB) in accordance with the manufacturer's guidelines. Standard digestions comprised 3 µl of 10X reaction buffer, approximately 500 ng DNA, 0.5 µl of restriction enzyme (equal to 10 U) and SDW to 30 µl. Reactions were incubated at 37°C for 1 hr and terminated by either heat-inactivation or by adding EDTA (10 mM), depending on manufacturer's recommendations.

2.4.5 Conversion of sticky-end to blunt ended DNA

Blunting of DNA fragments was accomplished using DNA Polymerase I, Large (Klenow) Fragment (NEB), following the manufacturer's guidelines. The enzyme utilises polymerisation and 3'→5' exonuclease activity to remove 3' overhangs and fill in 5' overhangs.

2.4.6 Dephosphorylation of DNA

Dephosphorylation of DNA was performed with calf intestinal alkaline phosphatase (CIAP), following the manufacturer's protocol (NEB), to prevent re-circularisation/re-ligation of DNA fragments during subsequent ligation stages (Section 2.4.7).

2.4.7 Ligation of DNA fragments

Ligation reactions used T4 DNA Ligase (Promega) in accordance with the manufacturer's guidelines with overnight incubation at 4°C. 1 µl of ligation product was used to transform One Shot[®] TOP10 Chemically Competent *E. coli*, as outlined by the manufacturer (Invitrogen).

2.4.8 Quantification of DNA

DNA concentration was determined using either a spectrophotometer (GeneQuant, Pharmacia Biotech) at a wavelength of 260 nm and calculated using $OD_{260} = 50 \mu\text{g ml}^{-1}$ double-stranded DNA (dsDNA; Sambrook *et al.*, 1989) or a NanoDrop (ThermoScientific).

2.4.9 Nucleotide sequencing of DNA

DNA sequencing was performed by Microsynth AG (Switzerland), Lark Technologies (UK) or Geneservice (UK).

2.4.10 Software and programs for data analyses

DNA sequences were edited and manipulated using Geneious software (Version 5.1; Biomatters Ltd) and analysed using web-based databases and programs including, National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/>), Cassava Online Archive (<http://cassava.psc.riken.jp/index.pl>), Phytozome (<http://www.phytozome.net/cassava>), The Arabidopsis Information Resource (TAIR; <http://www.arabidopsis.org/>), Genevestigator (<http://www.genevestigator.com>; Hruz *et al.*, 2008), PlantCARE (www.bioinformatics.psb.ugent.be/webtools/plantcare/html), peroxibase (<http://www.peroxibase.toulouse.inra.fr/>). Amplification primers were designed using the online NetPrimer package (www.premierbiosoft.com) and statistical analyses used SPSS (Version 18; SPSS, 2010).

2.4.11 Preparation of genomic DNA for Southern blot hybridisation

200 μl reactions comprised 20 μl 10X *Hind*III buffer (NEB), 4 μl of 100 mM spermidine, 4 μl *Hind*III (100 U μl^{-1} ; NEB), 0.2 μl RNase A (Invitrogen, Purelink™, 20 mg ml^{-1}), 20 μg DNA (Section 2.3.1) and SDW. The reactions were placed in sterile, thin-walled PCR tubes and incubated at 37°C overnight in a PTC-200 Peltier Thermal Cycler (MJ Research). To confirm complete digestion, 10 μl aliquots were electrophoresed in a TAE gel containing ethidium bromide (Section 2.4.2). To precipitate the DNA, 45 μl of 3 M NaOAc and 500 μl absolute ethanol were added to the tubes and incubated at -20°C for approximately 4 hr. The samples were centrifuged (16,100 x g) at 4°C for 30 min. The supernatant was removed and the samples air-dried for approximately 30 min. The pellet was gently resuspended in 30 μl SDW. Samples were co-electrophoresed with 1.5 μl digoxigenin (DIG)-labelled DNA Molecular Weight Marker III (Roche) in a TAE gel at 20V (Section 2.4.2).

2.4.12 Southern blotting

Electrophoresed agarose gels (Section 2.4.2) were soaked in 0.25 N HCl and gently agitated on a shaker for 30 min. The HCl was poured out, the gel rinsed briefly using SDW and soaked in denaturation solution (500 mM NaOH, 1.5 M NaCl) and returned to the shaker for 30 min. The denaturation solution was replaced with neutralisation solution (500 mM Tris, 1.5 M NaCl, 1 mM EDTA, pH7.2) for 30 min on the shaker. The DNA was transferred by capillary action (Southern, 1975) to a nylon membrane (Hybond N+, GE Healthcare) overnight using 20X SSC (3 M NaCl, 300 mM sodium citrate, pH7.6). The membrane was removed and the DNA covalently bound to the membrane using a CL-1000 Ultraviolet Crosslinker (UVP).

2.4.13 DIG hybridisation of Southern blot

Membranes were placed in a hybridisation tube containing 10 ml DIG Easy Hyb solution (Roche) and incubated (pre-hybridisation) for approximately 4 hr at 42°C. The solution was poured out and approximately 200 ng DIG-labelled probe (Section 2.4.14) was heated (100°C for 8 min) and added to 10 ml DIG Easy Hyb (pre-warmed to 42°C) before transfer to the hybridisation tube. The membrane was incubated overnight at 42°C, removed and washed three times in W1 (2X SSC, 0.1% (v/v) SDS) at room temperature for 5 min each. The membrane was then washed in W2 (0.2X SSC, 0.1% (v/v) SDS) at 68°C for 15 min (gently shaken) and then W3 (0.1X SSC, 0.1% (v/v) SDS) at 68°C for 15 min, shaking gently. The membrane was transferred to wash buffer (WB; Solution B1 (100 mM maleic acid, 150 mM NaCl, pH7.5) and 0.3% (v/v) Tween20) at room temperature for 3 min. WB was removed and replaced with 60 ml B2 (0.5 g blocking reagent (Roche) in 50 ml B1) and incubated (gently shaken) for 30 min. 5 µl anti-digoxigenin-AP Fab fragments (Roche) was added to 40 ml B2 and incubated on the membrane for 30 min. The membrane was washed twice with WB for 15 min each before a final wash for 1-2 hr. The membrane was incubated in 40 ml B3 (100 mM Tris-HCl pH9.5, 100 mM NaCl, 50 mM MgCl₂) for 5 min. 50 µl CDP-Star (Roche) was mixed in 5 ml B3 and repeatedly pipetted over the membrane for 10 min. Excess moisture was removed from the membrane using 3MM filter paper before it was wrapped in cling film and placed in a light-protected cassette with intensifying screens and autoradiograph film (Kodak, BioMax Light Film) and incubated at 37°C for approximately 15-30 min. Film was developed using a Curix 60 automatic developer (AGFA).

2.4.14 Preparation of DIG-labelled probe for hybridisation

A 50 µl reaction contained 2.5 µl of 10 µM *HygII* F primer (Table 2.1), 2.5 µl of 10 µM *HygII* R primer (Table 2.1), 5 µl buffer (NEB), 5 µl PCR DIG-Labeling Mix (Roche), 50 ng plasmid DNA containing *hptII* (e.g. pCambia 1305.1), 0.2 µl *Taq* Polymerase (NEB) and SDW. Samples were placed in sterile, thin-walled PCR tubes and cycled initially at 94°C (3 min), followed by 35 cycles of 94°C (1 min), 55°C (1 min), 72°C (1 min) and then 72°C (10 min). Products were resolved in a TAE gel (Section 2.4.2) and the correct sized fragment (approximately 400 bp) was isolated (Section 2.4.3). Eluted samples were stored at -20°C.

2.5 TISSUE CULTURE, TRANSFORMATION & MAINTENANCE OF CASSAVA

2.5.1 Generation of somatic embryos and friable embryogenic callus

Tissue culture and *Agrobacterium*-mediated transformation of friable embryogenic callus (FEC) from cassava cultivar TMS60444 was initially undertaken as previously described (Zhang & Gruissem, 2004; Zhang & Puonti-Kaerlas, 2004) and with guidance by P. Zhang (Shanghai Institutes for Biological Sciences, China). In summary, stem cuttings of *in vitro* TMS60444 plantlets were placed on cassava axillary medium (CAM; 1X MS salts with vitamins (Murashige & Skoog, 1962), 2% (w/v) sucrose, 2 µM CuSO₄, 10 mg L⁻¹ 6-benzylaminopurine (BAP), 0.3% (w/v) Gelrite™, pH 5.8) and incubated at 28°C (24 hr dark) for 4-6 d to enlarge the axillary meristems (i.e. buds). The buds were removed and transferred to cassava induction medium (CIM; 1X MS salts with vitamins, 2% (w/v) sucrose, 2 µM CuSO₄, 12 mg L⁻¹ picloram, 0.3% (w/v) Gelrite™, pH 5.8) and incubated at 28°C (24 hr dark) to induce formation of primary somatic embryos (Figure 2.2). After 2 weeks the embryogenic tissue was sub-cultured on fresh CIM and this procedure was repeated every two weeks to generate secondary somatic embryos. After approximately 8 weeks the somatic embryos were transferred to GD-based medium (1X GD salts with vitamins (Gresshoff & Doy, 1974), 2% (w/v) sucrose, 12 mg L⁻¹ picloram, 0.3% (w/v) Gelrite™, pH 5.8; incubate 28°C, 16 hr photoperiod) to induce the formation of FEC. After approximately 3-4 weeks the developing FEC were isolated from the embryos and incubated on fresh GD. Every 3 weeks the FEC were sub-cultured and non-embryogenic friable callus (NEFC) were removed to improve the purity of FEC. After approximately 6-9 weeks on GD media the FEC were transferred to SH liquid media (1X SH salts with vitamins (Schenk & Hildebrandt, 1972), 1X MS vitamins, 6% (w/v) sucrose, 12 mg L⁻¹ picloram, pH 5.8) that were incubated at 28°C, shaken at 100 rpm and 24 hr photoperiod. The media was replenished 2-3 times per week and sieved/filtered every 2

weeks for approximately 4-6 weeks to assist removal of NEFC and to aid proliferation (Figure 2.2).

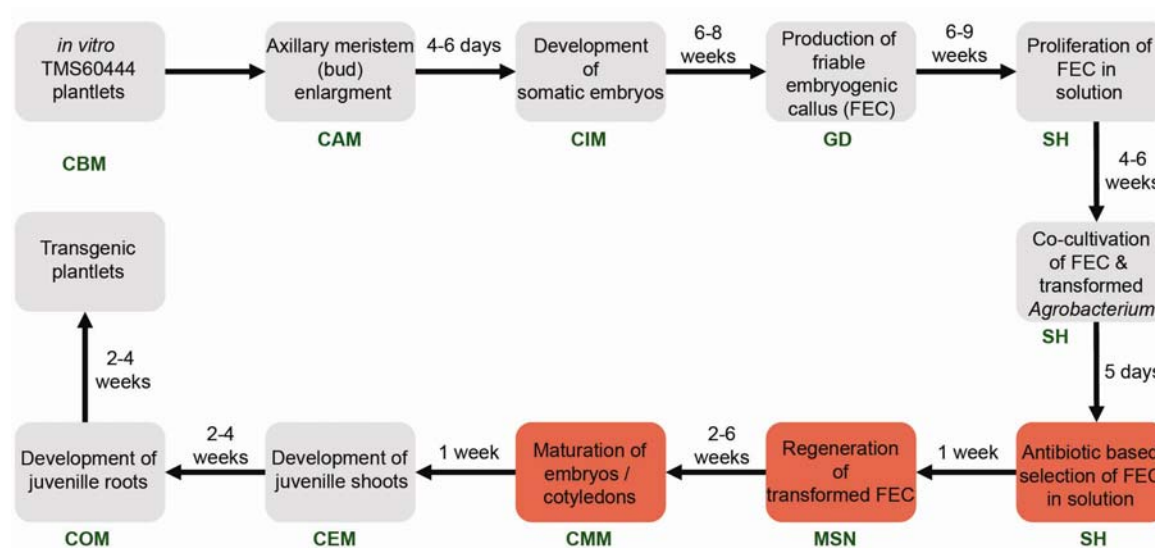


Figure 2.2 *Agrobacterium*-mediated transformation of FEC and regeneration of *in vitro* plantlets. Protocol as described by P. Zhang (Zhang & Grisse, 2004; Zhang & Puonti-Kaerlas, 2004) using cassava cultivar TMS60444. Media for each stage in green font; stages where hygromycin B antibiotic selection is used demarked by the red boxes.

2.5.2 *Agrobacterium*-mediated transformation of FEC and regeneration of embryos

The prepared FEC were inoculated with a suspension of *Agrobacterium* harbouring the target plasmid for approximately 5 d (Figure 2.2). Following this co-cultivation, the FEC were cultured in SH liquid media containing 25 mg L⁻¹ hygromycin B (henceforth referred to as hygromycin) to initiate selection of transformed tissue for approximately 1 week. FEC were spread on MSN media (1X MS salts with vitamins, 2% (w/v) sucrose, 1 mg L⁻¹ 1-naphthaleneacetic acid (NAA), 0.3% (w/v) Gelrite™, pH 5.8) supplemented with 25 mg L⁻¹ hygromycin and incubated at 28°C (16 hr photoperiod) to induce regeneration of embryos/cotyledons. Developing embryos appeared following 2-6 weeks incubation and were further cultured on cassava maturation medium (CMM; 1X MS salts with vitamins, 2% (w/v) sucrose, 2 µM CuSO₄, 0.1 mg L⁻¹ BAP, 0.3% (w/v) Gelrite™, pH 5.8; incubate 28°C, 16 hr photoperiod), cassava elongation medium (CEM; 1X MS salts with vitamins, 2% (w/v) sucrose, 2 µM CuSO₄, 0.4 mg L⁻¹ BAP, 0.3% (w/v) Gelrite™, pH 5.8; incubate

28°C, 16 hr photoperiod) and cassava shoot organogenesis media (COM; 1X MS salts with vitamins, 2% (w/v) sucrose, 2 μM CuSO_4 , 1 mg L^{-1} BAP, 0.5 mg L^{-1} indol-3-butyric acid (IBA), 4 mg ml^{-1} AgNO_3 , 0.3% (w/v) Gelrite™, pH 5.8; incubate 28°C, 16 hr photoperiod) to develop roots and shoots. Immature plant material was transferred to cassava basic medium (CBM; 1X MS salts with vitamins, 2% (w/v) sucrose, 2 μM CuSO_4 , 0.3% (w/v) Gelrite™, pH 5.8; incubate 28°C, 16 hr photoperiod) to develop *in vitro* plantlets (Figure 2.2). This material was subjected to a rooting test to screen for transgenic material and PCR amplification of antibiotic resistance genes (*hptII*) and transgenes (Section 2.1.2).

2.5.3 Transfer of *in vitro* cassava plantlets to soil

4 week old *in vitro* plantlets were gently extracted from their culture pots, washed in tepid water to remove media and planted in Levington's M2 compost mixed with perlite (3:1). Plants were covered with a vented lid and retained in a climate control room (28°C, 16 hr photoperiod) for 1 month prior to transfer to the glasshouse (28°C, >50% humidity) with supplementary lighting providing a 16 hr photoperiod for 4 months. Plants were fertilised with 1 g L^{-1} Vitax fertilizer twice weekly and watered daily, allowing the soil to become dry between watering. All transgenic material was maintained under conditions approved by the University of Bath Genetic Modification Safety Committee. The cassava tissue culture, transformation and plant propagation protocol is discussed in more detail in Chapter 4.

2.5.4 Harvesting of cassava plants, storage roots and PPD assays

Morphological data of harvested plants was catalogued and storage roots were used for RNA extraction (Section 2.6.1) and PPD assays. Plant height was measured (cm) from the root stock to the apical growth tip/leaf. Soil was washed from the intact roots and the root stock was removed (cutting the stem approximately 2 cm above the base of the stem) and weighed (g). Simultaneously, the youngest fully expanded leaf and the 5th leaf down were isolated, wrapped in aluminium foil and frozen in liquid nitrogen. Roots greater than approximately 1 cm in diameter were removed. A slice from the central region of the root was taken, the bark discarded and then grated into a mortar containing liquid nitrogen (Figure 2.3). The tissue was ground to a fine powder and transferred to a pre-cooled, sterile 15 ml plastic tube and stored at -80°C. This slice/section represents 0 hr post-harvest. A thin slice (approximately 5 mm) was removed from the extremities of the two remaining halves and placed interior side down on a sterile Petri dish. The root

samples were stored in a covered plastic box at 26°C. At the appropriate time point (24 hr and 72 hr or 96 hr) a root cutting was removed and the dry tissue was skimmed from the upper end. Two slices (approximately 5 mm each) were taken and the inner face was photographed (Figure 2.3). The bark was removed and samples were ground to a fine powder in liquid nitrogen using a pestle and mortar. Samples were transferred to a pre-cooled, sterile 15 ml plastic tube and stored at -80°C. Pestle and mortars were baked at 200°C for 6 hr and then flame-sterilised prior to use.

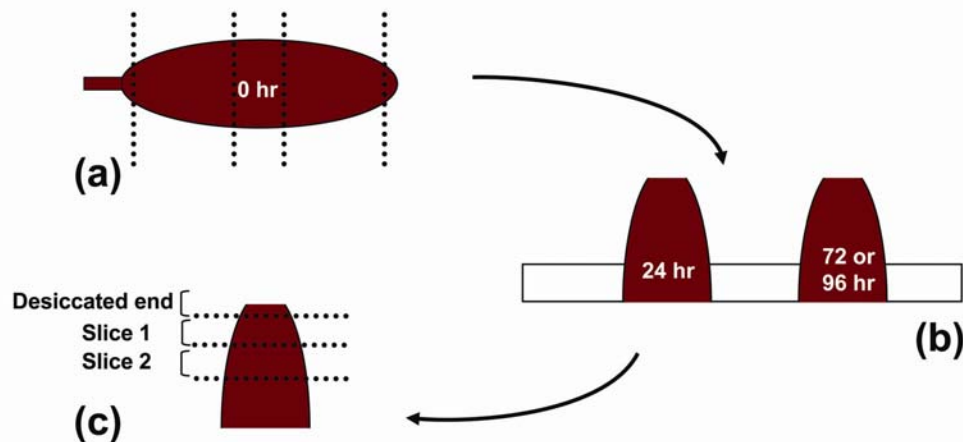


Figure 2.3 Method for assessing PPD in glasshouse-cultivated cassava storage roots.

Intact storage root (approximately >1 cm diameter) isolated from root stock and sectioned into three. Central section represents 0 hr time point post-harvest (a). Remaining two sections placed on sterile Petri dish and incubated (26°C, dark). After 24 hr and 72 hr or 96 hr (b) a sample is removed, the desiccated end skimmed off (approximately 5 mm of tissue) and two sections are cut, each approximately 5-10 mm thick (c). Photograph taken of the inner face of each section to assess PPD. Dotted lines indicate approximate cutting positions. Diagram not to scale.

2.6 RNA EXTRACTION AND MANIPULATION

2.6.1 RNA extraction from cassava storage roots and leaves

Approximately 0.5 g of frozen, powdered tissue (Section 2.5.4) was transferred to a pre-cooled (stored on ice) sterile 15 ml tube and approximately 6 ml pre-warmed (50°C) extraction buffer was added (2% (w/v) CTAB, 2% (w/v) PVP-40, 100 mM Tris-HCl, 25 mM EDTA, 2 M NaCl, 0.5 g L⁻¹ spermidine and 2% (v/v) β-mercaptoethanol, added prior to use). Samples were mixed and incubated at 50°C for 15 min. An equal volume of chilled (4°C) chloroform:isoamylalcohol (24:1; Sigma-Aldrich) was added and the

samples mixed. Tubes were centrifuged at 2790 x g for 10 min at 4°C. The supernatant was transferred to a sterile 15 ml tube and an equal volume of chloroform:isoamylalcohol (24:1) added. Samples were mixed and centrifuged as previously. 1 ml of the supernatant was aliquoted into sterile 1.5 ml microfuge tubes containing 315 µl 8 M LiCl (Sigma-Aldrich), inverted until mixed and incubated at 4°C overnight. Samples were centrifuged (16,100 x g) for 20 min at 4°C, the supernatant removed and pellet air-dried for approximately 10 min by inverting the tube on sterile tissue. Pellets were resuspended in a total of 70 µl NFW for DNase treatment (Section 2.6.2).

2.6.2 DNase treatment of RNA samples

70 µl of total nucleic acid sample (Section 2.6.1) was gently mixed with 10 µl TURBO™ buffer, 17 µl NFW and 3 µl TURBO™ DNase (Ambion) in a 0.2 ml sterile, thin-walled PCR tube. Samples were incubated at 37°C for 30 min in a PTC-200 Peltier Thermal Cycler (MJ Research) before being cleaned-up using the Plant Mini RNA Extraction kit as directed by the manufacturer (Qiagen). RNA was eluted in 30 µl NFW and stored at -80°C.

2.6.3 RNA quantification

1 µl of extracted RNA sample (Section 2.6.2) was quantified and the integrity determined using the Experion™ RNA StdSens Analysis kit (Bio-Rad Laboratories) – a chip based microfluidics automated electrophoresis system. RNA was used immediately for cDNA synthesis (Section 2.6.4).

2.6.4 cDNA synthesis (reverse-transcription PCR; RT-PCR)

cDNA was synthesised using 1 µg of RNA (Sections 2.6.2 and 2.6.3), oligo(dT)₂₀ and SuperScript™III First-Strand Synthesis SuperMix as directed by the manufacturer (Invitrogen). cDNA was stored at -20°C. For each biological sample, two cDNA synthesis reactions were performed serving as technical replicates for analysis (Section 2.1.3).

2.7 BIOCHEMICAL & HPLC TECHNIQUES

2.7.1 Total protein extraction from cassava

An equal volume of frozen, powdered tissue (Section 2.5.4) was mixed with extraction buffer (50 mM HEPES buffer, 2 mM sodium metabisulphite, pH7.2). The homogenate was transferred to a sterile 2 ml microfuge tube and centrifuged (10,000 x g) at 4°C for

30 min. The supernatant was transferred to a sterile microfuge tube and centrifuged as previously. The supernatant was stored at -20°C.

2.7.2 Bradford assay

The Bradford assay was performed to determine the total protein content of samples. 250 µl of Bradford reagent (Sigma-Aldrich) and 5 µl protein sample (Section 2.7.1) were gently mixed in a 96-well plate (NUNC) and incubated in the dark at room temperature for 30 min. Bovine serum albumin (BSA; NEB) standards (0.1 – 1.4 mg ml⁻¹) were prepared. Absorbance (595 nm) was measured using an Omega Microplate Reader (BMG) and a standard curve generated using the control samples. Total protein content (mg ml⁻¹) of the test samples was determined.

2.7.3 Ascorbate peroxidase (APX) enzyme assay

APX enzyme activity was assayed using a spectrophotometric method *via* the rate of guaiacol oxidation (tetraguaiacol formation; Chance & Maehly, 1955). The assay measures the activity of both APX and guaiacol peroxidases since both enzymes can use guaiacol (2-methoxyphenol, an organic compound that darkens during oxidation) as an electron donor (Mehlhorn *et al.*, 1996). 950 µl APX assay buffer (50 mM NaOAc, 15 mM guaiacol) was placed in a plastic cuvette. 10 µl of buffer (50 mM HEPES buffer (pH 7), 2 mM sodium metabisulphite) was added and the sample loaded into a Cary 50 recording spectrophotometer (Varian; A₄₇₀). Readings were taken every 0.5 sec and temperature maintained at 30°C. The 10 min run was started and 40 µl of 240 mM H₂O₂ added. A₄₇₀ *versus* time was plotted and the gradient of the line determined to calculate the rate of reaction. This procedure was repeated with pure horseradish peroxidase (HPX) enzyme to generate a standard curve. Root extracts (Section 2.7.1) were then tested and A₄₇₀ readings converted to units of APX based on the standard curve. Two technical replicates were used for each standard control and experimental sample. Samples were standardised based on their total protein content (Section 2.7.2) to account for variation in protein extraction efficiency and pipetting differences between samples.

2.7.4 Tissue preparation for determination of non-protein thiols

Approximately 200 mg of frozen, powdered tissue (Section 2.6.1) was mixed with 2 ml 0.1 N HCl (828 µl concentrated HCl in 99.17 ml NFW) in a 2 ml microfuge tube (Eppendorf, PCR Clean range) and stored on ice. Samples were centrifuged (16,100 x g)

for 10 min at 4°C. The supernatant was transferred to a sterile 2 ml microfuge tube and centrifuged as previously. Supernatants were stored at -80°C. For each sample a dithiothreitol (DTT) treated and non-treated preparations are required. For DTT treatment, which allows measurement of oxidised and reduced glutathione (GSSG and GSH, respectively), 25 µl of supernatant was mixed with 25 µl of 0.1 M NaOH and 1 µl of 0.1 M DTT and the mix was incubated in the dark at 37°C for 15 min. For non-treated samples (which results in measurement of GSH only) 25 µl supernatant was mixed with 25 µl of 0.1 M NaOH. Both DTT- and non-treated samples were derivatised by mixing 50 µl of the neutralised extract with 35 µl SDW, 10 µl of 1 M Tris-HCl (pH 8.0) and 5 µl of 10 mM monobromobimane (MB, prepared in 100% acetonitrile; Newton *et al.*, 1981) for non-treated samples or 5 µl of 100 mM MB for DTT treated samples. The solutions were mixed and incubated in the dark at 37°C for 15 min. 100 µl of 9% (v/v) acetic acid was added to the samples, mixed and loaded onto a Costar spin-X 0.22 µm spin filter. Tubes were centrifuged for 4 min at 16,100 x g. 200 µl of elution was transferred to a HPLC sample vial and 80 µl was injected into the HPLC - a Waters 2690 Alliance HPLC with spherisorb C18.ODS2 4.6 X 250 mm column, 5 µm packing with 1 cm guard cartridge. Flow rate of 1 ml min⁻¹ and utilised buffer B (100% acetonitrile) and buffer C (2.5 ml acetic acid, 0.5 ml 3 M KOH in 1 L SDW, pH3.2). Detection *via* a Waters 474 fluoromonitor with excitation at 390 nm and emission at 482 nm.

2.7.5 Preparation and derivatisation of standards for HPLC

To obtain quantitative data it is necessary to generate known standards of GSH, cysteine and γ -glutamylcysteine (γ -EC). 15 mM stocks of each standard were prepared in 0.1 N HCl and stored at -20°C. Concentration of standards was assessed *via* a 5,5'-dithiobis-(2-nitrobenzoic acid; DTNB) assay (Sigma-Aldrich). 50 µl of each 1 mM GSH, γ -EC and cysteine were further mixed together with 350 µl of 0.1 N HCl to generate a combined stock of thiol (0.1 mM). 25 µl of this stock was neutralised by adding 25 µl of 100 mM NaOH and mixed with 35 µl SDW, 10 µl of 1 M Tris-HCl (pH 8.0) and 5 µl of 10 mM MB. Stock was incubated in the dark at 37°C for 15 min. 900 µl of 5% (v/v) acetic acid was added, mixed and 500 µl was loaded onto a Costar Spin-X 0.22 µm spin filter, centrifuged for 4 min at 13,000 x g in a centrifuge. 200 µl of the elution was transferred to a HPLC sample vial. 10 µl (0.025 nmol), 20 µl (0.05 nmol), 40 µl (0.1 nmol) and 50 µl (0.125 nmol) were injected into the HPLC.

3 CREATION OF EXPRESSION CASSETTES FOR CASSAVA TRANSFORMATION

3.1 INTRODUCTION

Plant transformation utilises vector systems for the integration and expression of transgenes in the plant host genome. The delivery of target DNA can be achieved using various techniques, including *Agrobacterium*-mediated transformation, microparticle bombardment (usually referred to as biolistic inoculation), electroporation and chemical mediated (e.g. polyethylene glycol) transformation (Barampuram & Zhang, 2011; Rao *et al.*, 2009; Shewry *et al.*, 2008). *Agrobacterium*- and biolistic-mediated transformation are predominately employed in cassava biotechnology (Schöpke *et al.*, 1996; Zhang *et al.*, 2000a), although the latter technique has a propensity to introduce multiple copies of the transgene and superfluous vector DNA, which may be deleterious to gene expression and undesirable in transgenic crops for commercialisation (Taylor & Fauquet, 2002). *Agrobacterium*-mediated transformation, however, has proven to be more reliable in integrating single copies of the transgene with consistent expression over generations and is the favoured technique of the Plant Biotechnology Group (ETH Zürich, Switzerland) where cassava transformation for this investigation was performed. Due to the absence of a suitable commercially or freely available expression cassette, an *Agrobacterium* compatible vector was modified with features specific to the project requirements i.e. a root-specific promoter to drive transgene expression, selectable marker genes (e.g. antibiotic resistance gene and visual reporter gene) and an appropriate cloning system.

3.1.1 Binary/expression cassettes and *Agrobacterium* strains

There is a medley of binary vectors available for plant transformation (Lee & Gelvin, 2008) that have become increasingly sophisticated since the introduction of pBIN (Bevan, 1984). Amongst the most widely used are the pGreen (Hellens *et al.*, 2000), pCAMBIA (www.cambia.org) and Gateway® vectors (Karimi *et al.*, 2007; Karimi *et al.*, 2002). In cassava transformation, vectors including pHMG (Zhang *et al.*, 2000b), pILTAB313 (Schöpke *et al.*, 1996), pBI121 (Ihemere *et al.*, 2006), pPZP111 and pCAMBIA 2301 (Jørgensen *et al.*, 2005) and also the RNAi construct pRNAi-dPro (Vanderschuren *et al.*, 2007) have all been used successfully. Most of these vectors are

the result of modification and indeed the range of pCAMBIA vectors are themselves based on pPZP, which were developed for their small size and stability in *Agrobacterium* (Gelvin, 2009; Hajdukiewicz *et al.*, 1994). Similarly for vectors, there is a plethora of non-pathogenic/disarmed *Agrobacterium* strains harbouring the *vir* helper plasmids required for replication and T-DNA transfer from a binary vector (Gelvin, 2009; Lee & Gelvin, 2008). Li *et al.* (1996) compared strains LBA4404 (Ooms *et al.*, 1982), C58C1 and EHA105 (Hood *et al.*, 1993) each carrying *uidA*-containing plasmids (Section 3.1.3) for their ability to transform somatic cotyledons of cassava. LBA4404 gave the highest transient expression rate and has subsequently become the favoured strain by many different research groups undertaking cassava transformation (Ihemere *et al.*, 2006; Vanderschuren *et al.*, 2007; Zhang *et al.*, 2003b).

3.1.2 Marker genes to screen transformed plant material

Selectable marker genes located in the T-DNA are pivotal in the identification of transformed plant material. Surprisingly few cells integrate the target DNA, which is not a problem in transformation of plant species such as *Arabidopsis* where large numbers of seeds can be easily screened, but in recalcitrant species the poor efficiency limits available transgenic material. Over 50 classical selectable marker genes have been documented (Miki & McHugh, 2004; Sundar & Sakthivel, 2008) including *bar* that encodes phosphinotricin acetyl transferase, which imparts resistance to the herbicide Basta and has been used to generate transgenic cassava (Sarria *et al.*, 2000). However, antibiotic resistance genes are most commonly used, such as *nptII* (encoding neomycin phosphotransferase; Fraley *et al.*, 1983) and *hptII* (encoding hygromycin phosphotransferase; Waldron *et al.*, 1985) that prevent disruption of protein synthesis in transformed tissue by aminoglycoside antibiotics (e.g. kanamycin, paramomycin and geneticin) and hygromycin, respectively. Interestingly, hygromycin is reported to negatively impact on the regeneration of cassava tissue cultured material (Schöpke *et al.*, 1996; Schreuder *et al.*, 2001), yet this stringency ensures less non-transformed material develops if optimal concentrations are used (Schreuder *et al.*, 2001; Zhang & Puonti-Kaerlas, 2004). Transgenic cassava have been successfully generated using both *hptII* (Schreuder *et al.*, 2001; Zhang & Gruissem, 2004; Zhang *et al.*, 2003b) and *nptII*-containing plasmids (Chellappan *et al.*, 2004; Jørgensen *et al.*, 2005). Significantly, pCAMBIA supply a range of vectors with both *hptII* and *nptII* that can be modified without encountering complications with intellectual ownership rites.

3.1.3 Visual reporter genes for identification of transformed material

Visual reporter genes are important for monitoring transformation progress, confirming identification of transgenic material and also for studying gene expression patterns. Raemakers *et al.* (1996) and Munyikwa *et al.* (1998) utilised the luciferase (*luc*) gene isolated from firefly (Ow *et al.*, 1986) to screen tissue cultured material and mature cassava plants following treatment with the substrate luciferin. Alternatively, Schöpke *et al.* (1996), Li *et al.* (1996) and Zhang & Gruissem (2004) all used constructs containing the *E. coli uidA* reporter gene that encodes β -glucuronidase (*gusA* or GUS) and produces a blue/black precipitate following the addition of an appropriate substrate (e.g. 5-bromo-4-chloro-3-indoxyl- β -D-glucuronic acid, cyclohexylammonium salt; X-gluc) (Jefferson *et al.*, 1987). The green fluorescent protein (GFP) originally isolated from jellyfish (Chalfie *et al.*, 1994) has also been used in cassava transformation (N. Taylor, *pers. comm.*; Taylor *et al.*, 2004). The screening of material with fluorescence marker genes such as *luc* and *gfp* is non-destructive unlike the assay for GUS activity. However, the latter approach is less labour-intensive and does not require the experience and equipment needed for isolating tissue transformed with fluorescence marker genes. pCAMBIA 1305.1 (NCBI accession AF354045; Figure 3.1) contains the synthetic *GUSPlus* gene derived from *Staphylococcus* sp. sequence and the produced GUSPlus is up to ten times more detectable than the conventional *E. coli* GUS (Broothaerts *et al.*, 2005; pCAMBIA, 2011).

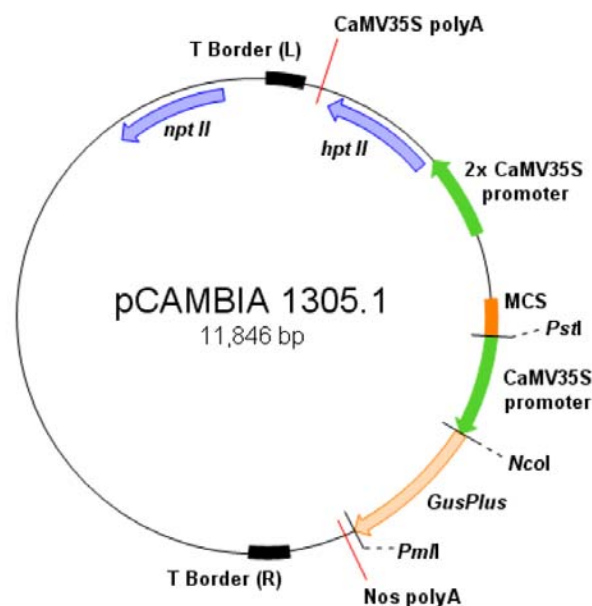


Figure 3.1 Simplified map of pCAMBIA 1305.1. Features include the *CaMV35S* promoter (Odell *et al.*, 1985) regulating expression of the *GUSPlus* reporter gene and hygromycin antibiotic

resistance gene (*hptII*; blue arrow); T-DNA borders (depicted by black boxes); the multiple cloning site (MCS; orange box); positions of *nos* and *CaMV35S* polyA regions are also marked (red lines). The genes required for survival in bacteria have been omitted for simplicity, with the exception of the *nptII* resistance gene (blue arrow). Figure provided by M. T. Page (University of Bath).

3.1.4 Cloning strategy

Target genes are cloned into pCAMBIA 1305.1 using restriction enzyme digestion and DNA ligation techniques - a relatively simple approach but one that is time consuming and potentially problematic when cloning several genes. A prominent complication is the necessity to identify unique enzyme recognition sites in the correct positions that are lacking in the selected promoter and transgene to prevent fragmentation during digestion. However, in recent years the Gateway® cloning system (Invitrogen) has been developed that utilises the site-specific recombination properties of bacteriophage lambda (Karimi *et al.*, 2007; Landy, 1989). The target sequence is flanked (*via* PCR) with the appropriate lambda sequence, known as *attachment* or *attB* sites, to allow recombination at specific sites (*attP*) in an intermediate or donor vector (pDONR™). This process is colloquially known as the BP reaction (Figure 3.2a). Further recombination between the *attL* sites in the product (entry clone) and the *attR* sites in the expression or destination vector transfers the target sequence (the LR reaction), creating the desired final expression clone (Figure 3.2b). Both the BP and LR reactions require the involvement of bacteriophage lambda and *E. coli* enzymes, provided as BP clonase™ and LR clonase™ (Invitrogen). The presence of *ccdB* – a bacterial suicide gene – in the host vector is used to screen for successfully recombined plasmids. The necessary infrastructure to adapt a binary vector to be Gateway® cloning compatible is available from Invitrogen (Karimi *et al.*, 2002).

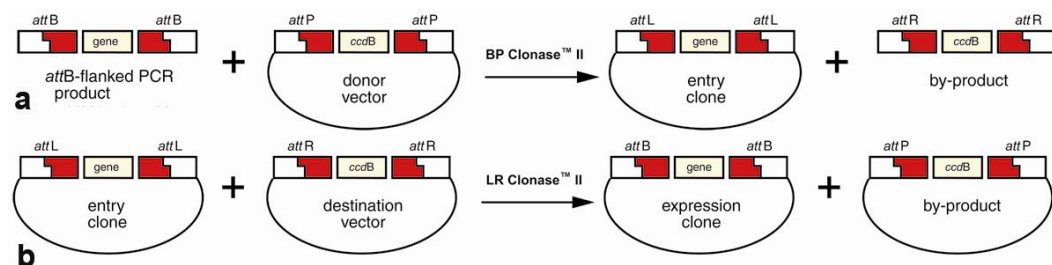


Figure 3.2 Overview of Gateway® cloning. *att* sites (*attB*) are added to the target sequence *via* PCR and the product mixed with the donor vector. Recombination of the *attB* and vector-based *attP* sites, catalysed by BP clonase™ mix, results in the integration of the gene to form the entry

clone **(a)**. The newly formed *att* sites (*attL*) in the entry clone recombine with *attR* sites in the destination vector, catalysed by LR clonase™ mix, yielding the final expression construct **(b)**. The *ccdB* gene allows screening of recombined plasmids. Figure adapted from www.invitrogen.com.

3.1.5 Selection of a promoter for transgene expression

Promoters used in plant biotechnology are traditionally divided into three categories; constitutive (active continuously in all tissues), spatiotemporal (tissue or developmental specific) and inducible (regulated by an external signal or chemical; Potenza *et al.*, 2004). To date only a few promoters have been isolated from cassava including the regulatory region from *C15*, which has sequence similarities to cytochrome P450 proteins and *C54* that is considered to be related to a glutamic acid-rich protein (Pt2L4). Translational fusions to *uidA* showed expression predominately in the starch-rich parenchyma cells of transgenic cassava storage roots with some expression in vascular tissue (Zhang *et al.*, 2003a). *MecPX3* is a putative secretory peroxidase from cassava and was of particular interest due the gene expression profile (Reilly *et al.*, 2007), but unfortunately the regulatory sequence remained uncharacterised (please see Chapter 7 for further details). Although little is known about cassava gene promoters, the use of endogenous sequence to regulate transgene expression remains a contentious issue given the possibility for gene silencing (Kooter *et al.*, 1999; Vaucheret *et al.*, 1998) and to what extent this may arise in cassava has not been examined. Importantly, the chosen promoter should be root/tuber specific and/or closely associated with PPD to ensure optimal gene expression. Various heterologous promoters have been adopted for transgene expression in cassava, including potato *GRANULE-BOUND STARCH SYNTHASE 1* (*GBSS1*) and *PATATIN* used for altering starch content and production in storage roots (Ihemere *et al.*, 2006; Raemakers *et al.*, 2005). Furthermore, *PATATIN* (*PS20*) promoter-*uidA* fusions revealed GUS activity in potato tubers was approximately 500-fold greater than in leaves and 5,000-fold greater compared to roots (Wenzler *et al.*, 1989). Even in *Arabidopsis* - a species that clearly lack tubers or storage roots – GUS production was highly root specific although some expression was observed in the leaves (Martin *et al.*, 1997). The characteristics of *PATATIN* promoters therefore make them suitable candidates for investigations into PPD of cassava.

3.1.5.1 Gene function and promoter characteristics of *PATATIN*

PATATIN is a family of 40 kDa glycoproteins that function as the primary storage protein in potato tubers (*S. tuberosum*). They also serve as a lipid acyl hydrolases, cleaving fatty

acids in membranes in response to wounding of the tuber and enhancing suberisation for plant defence (Racusen & Foote, 1980; Shewry, 2003). The first nucleotide sequences of intact, functional *PATATIN* genes from potato were provided during the 1980s and comprise *PAT21* (Bevan *et al.*, 1986), *PS20* (Mignery *et al.*, 1988) and *B33* (Rocha-Sosa *et al.*, 1989). cDNA and Northern blot analyses revealed that two major transcripts of *PATATIN* exist (each approximately 1,500 nucleotides in length) that were differentiated based on nucleotide sequence and expression profile. Class I transcripts lack a 22 nucleotide insertion in the 5'-untranslated region (5'-UTR) and are tuber specific, whereas class II transcripts contain the additional sequence, are 100-fold less abundant in tubers and instead expressed primarily in roots (Mignery *et al.*, 1984; Mignery *et al.*, 1988; Pikaard *et al.*, 1987). Although there is heterogeneity amongst transcripts, the 5' flanking sequence of both class I and II are highly homologous until bp position -87 and then diverge. The conserved region contains the TATA, CAAT and core enhancer (CE) elements (Mignery *et al.*, 1988; Twell & Ooms, 1988; Figure 3.3). *PATATIN* expression accompanies tuberisation (Stupar *et al.*, 2006) but exogenous sucrose can also stimulate expression in non-tuber tissue such as leaves and stems, although sucrose is not believed to be directly responsible for gene expression (Grierson *et al.*, 1994; Rocha-Sosa *et al.*, 1989). A common feature of class I *PATATIN* promoters is the highly conserved 100 bp region containing the so-called A-box and B-box elements that are critical for gene expression (Grierson *et al.*, 1994; Jefferson *et al.*, 1990; Figure 3.3). This region is located within the minimal promoter (up to position -344 bp) and was identified using truncated promoter-*uidA* fusions. A+B-box repeats have also been identified at more distal positions (e.g. -582 bp in *PAT21* and -809 in *B33*) and have also been implicated in gene expression (Liu *et al.*, 1990; Liu *et al.*, 1991). Interestingly, deletions in a conserved 10 bp motif (GCTAAACAAT) within the B-box of *PAT21* (within the minimal promoter region) led to a reduction in both tuber specificity and sucrose-inducible gene expression in transgenic potato. Furthermore this research led to the identification of the STOREKEEPER protein that binds within the B-box and is hypothesised to regulate *PATATIN* expression in potato tubers (Zourelidou *et al.*, 2002).

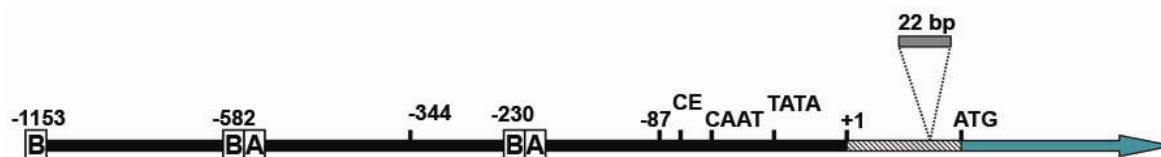


Figure 3.3 Typical Class I *PATATIN* promoter. Information based on *PAT21* promoter (Bevan *et al.*, 1986) showing translation initiation site (ATG), 5'-UTR (hatched area) and transcription start (+1). Approximate location of 22 bp sequence, absent in class I but present in class II *PATATIN* promoters. Class I and Class II have high sequence homology until bp position -87, the region containing putative TATA, CAAT and core enhancer (CE) elements. A-box and B-box elements (100 bp in length) and the minimal promoter region to bp -344 shown. Diagram not to scale.

3.2 RESEARCH OBJECTIVES

The objective is to create expression constructs containing sense and antisense genes, previously identified to modulate ROS, for *Agrobacterium*-mediated transformation of cassava. To achieve this the pCambia 1305.1 binary vector will be modified to integrate a class I *PATATIN* promoter (termed here as *StPAT*) and also the necessary components to clone target sequence using Gateway® technology. For simplicity, the techniques and results will be described together in this chapter.

3.3 METHODS & RESULTS

3.3.1 PCR isolation and sequencing of the *PATATIN* promoter

The *PATATIN* promoter (*StPAT*) was PCR amplified (Section 2.1.1) from the vector (provided of P. Zhang; Shanghai Institutes for Biological Sciences, China) using primers Pat-PstI F and Pat-Pml R (Table 2.1), which contain the restriction enzyme recognition sites for *PstI* and *PmlI*, respectively. The product (approximately 1,020 bp) was observed following agarose gel electrophoresis (Section 2.4.2) and isolated (Section 2.4.3) for cloning into the pCR®2.1-TOPO® TA vector and transformation of One Shot® TOP10 chemically competent *E. coli* (Section 2.2.1). Following sub-culturing of selected colonies and plasmid isolation (Section 2.2.6), vector specific primers were used to confirm the nucleotide sequence (Section 2.4.9). The cloned fragment was subsequently digested from the TA vector using restriction enzymes *PstI* and *PmlI* (Section 2.4.4). Agarose gel electrophoresis was used to visualise that the appropriate sized product had been isolated in readiness for ligation into the pCambia vector.

3.3.2 Restriction enzyme digestion of pCAMBIA 1305.1 and ligation of StPAT promoter

The pCAMBIA 1305.1 vector was digested by restriction enzymes *Pst*I and *Pml*I (Section 2.4.4), removing the LacZ alpha fragment, *CaMV*35S promoter, catalase intron and *GUS*Plus (Figure 3.4a). The products were electrophoresed in an agarose gel to ensure the unwanted fragment (approximately 2,818 bp) had been excised. The desired fragment (linearised vector, approximately 9,028 bp) was then isolated from the gel (Section 2.4.3), treated with CIAP (Section 2.4.6) to minimise the possibility for ligation of the excised fragment and re-circularisation in subsequent reactions. The *Pst*I and *Pml*I recognition sites were selected in part due to the fact that neither is present in the StPAT promoter. The linearised vector (Figure 3.4b) and isolated StPAT promoter (Section 3.3.1; Figure 3.4c) were ligated and the mix was used to transform One Shot[®] TOP10 Chemically Competent *E. coli*, which were plated on LB agar media supplemented with 50 µg ml⁻¹ kanamycin. Plasmid DNA was extracted from a selection of colonies (Section 2.2.6) and the presence of the intermediate vector (termed pCAM:PAT:INTER) was confirmed *via* sequencing using primers CAMBSeqF1 (anneals upstream of the MCS) and DESTSeqR1 (anneals downstream of the right T-DNA border; Table 2.1; Section 2.4.9), thus amplifying across the ligation boundaries.

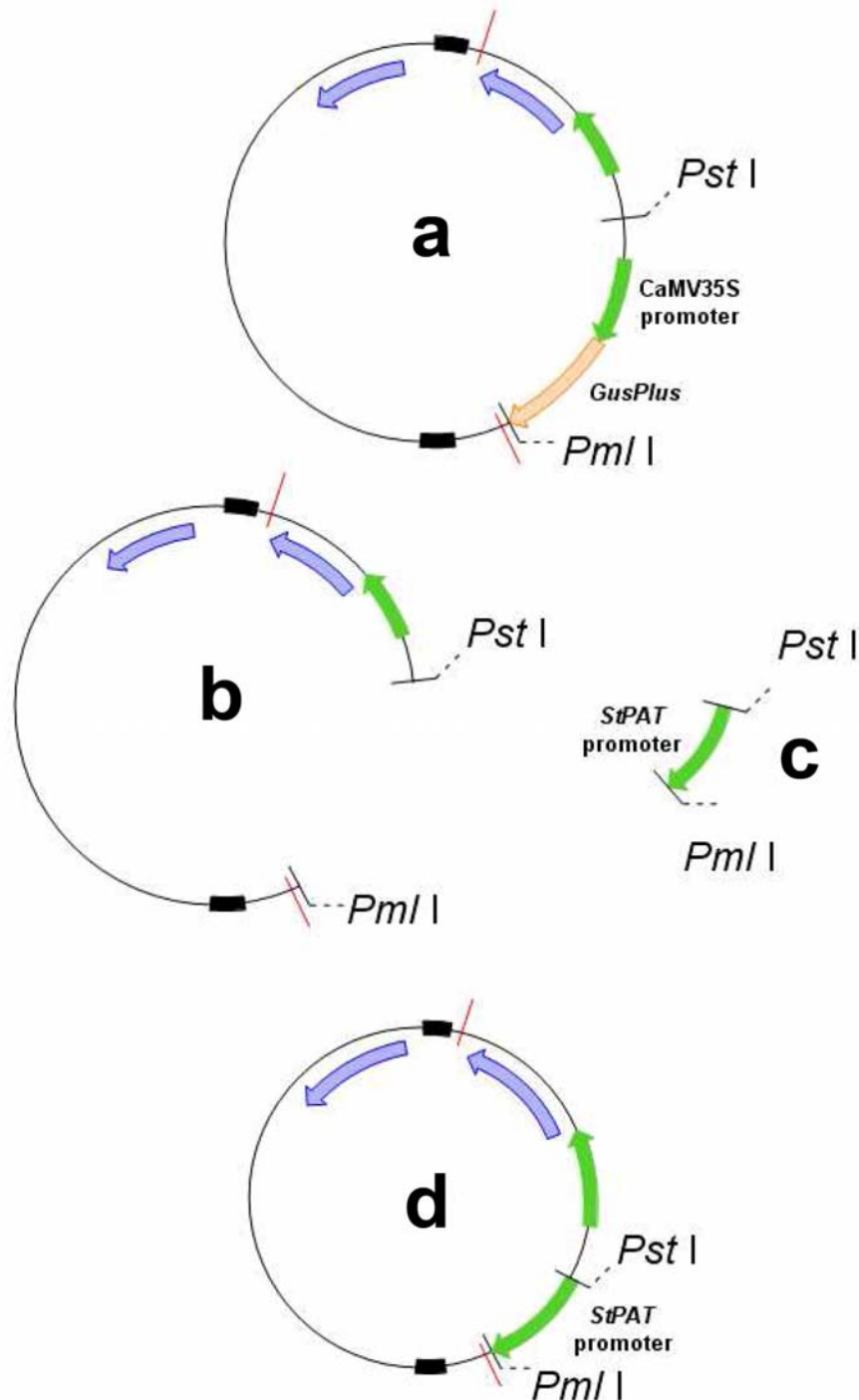


Figure 3.4 Modification of pCambia 1305.1 to incorporate the *StPAT* promoter. Restriction digestion of pCambia 1305.1 by *Pst*I and *Pml*I (a), generating a linearised vector (b) for ligation of the *StPAT* promoter (c). The intermediate vector (d) is referred to as pCAM:PAT:INTER. Promoters (green arrows), antibiotic resistance genes (blue arrows), polyA positions (red lines) and the T-DNA borders (black boxes) are shown. Relevant restriction enzyme recognition sites indicated. Figure provided by M. T. Page (University of Bath).

3.3.3 Conversion of pCAM:PAT:INTER to be Gateway® compatible

The adaptation of a vector to enable Gateway® cloning requires a blunt-end restriction site in the correct location for insertion of the Gateway® cassette. The cassette (supplied as part of the Gateway® Vector Conversion System; Invitrogen) comprises the recombination sites required for insertion of genes from entry clones (Section 2.2.3), as well as the *ccdB* and chloramphenicol resistance gene. pCAM:PAT:INTER (Figure 3.5a) was digested with *Pml*I (a blunt-ending restriction enzyme) to linearise the vector immediately downstream of the *St*PAT promoter. The vector was subsequently treated with CIAP to remove the 5' phosphates and prevent re-circularisation (Section 2.4.6; Figure 3.5b). The selected Gateway® reading frame cassette (rfA; Figure 3.5c) was ligated into the vector and the mix was used to transform One Shot® Omnimax 2-T1 Chemically Competent *E. coli* (Invitrogen), which are resistant to the negative impact of the *ccdB* gene. Extracted plasmid DNA (Section 2.2.6) was digested using *Bsr*GI, whose enzyme recognition site is specific to Gateway®-related sequences (Section 2.4.4). The products were electrophoresed (Section 2.4.2) and compared to the expected banding pattern (10,066 bp, 1,283 bp and 402 bp; data not shown). The vector was partially sequenced using primer DESTSeqF1, which binds in the *St*PAT promoter 137 bp upstream of the insertion site of the rfA Gateway® cassette, to ensure it was in the correct orientation (Table 2.1; Section 2.4.9; Figure 3.5d).

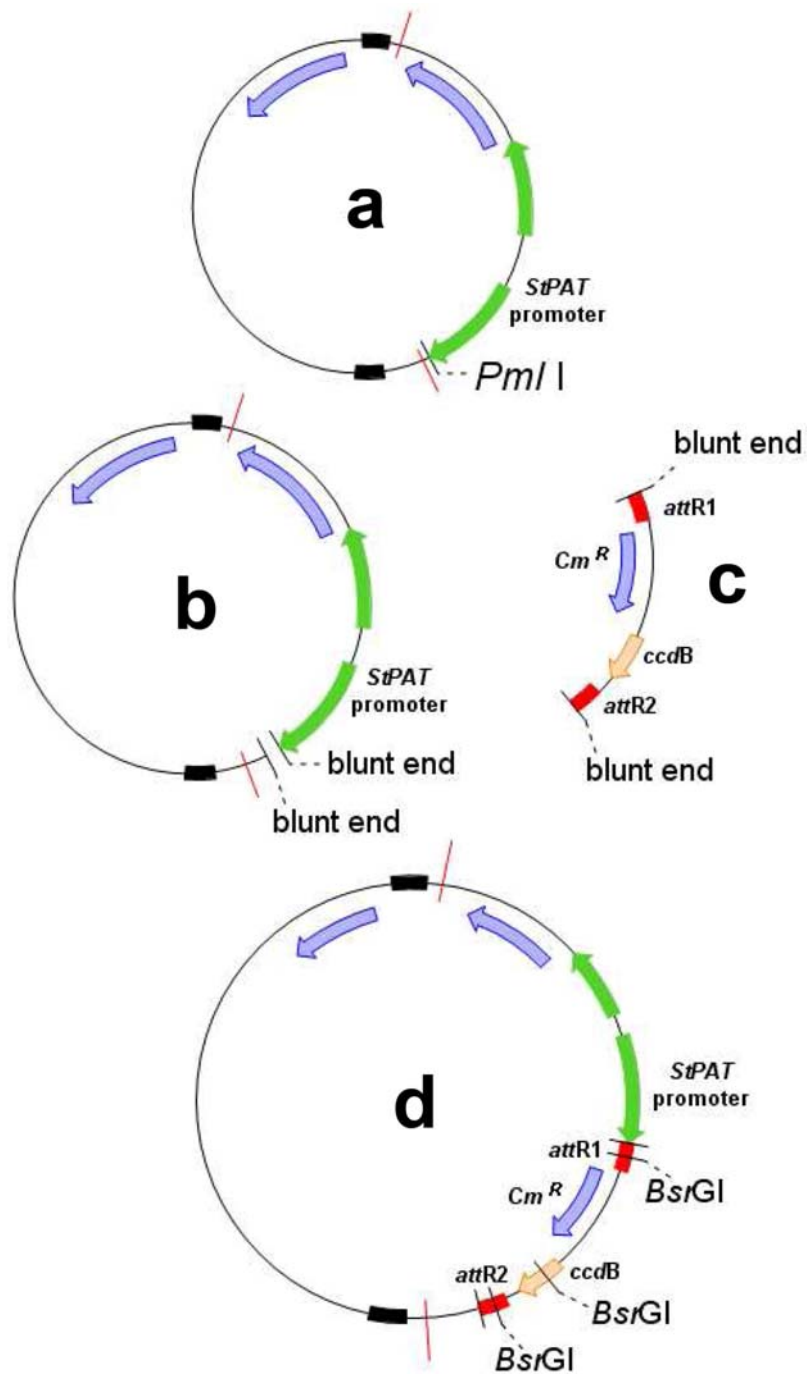


Figure 3.5 Conversion of pCAM:PAT:INTER into a Gateway[®] compatible expression construct. Restriction enzyme digested with *Pml*I (a) to generate blunt ends immediately downstream of StPAT promoter (b). Gateway[®] Reading Frame Cassette rfA (Invitrogen) (c) was ligated into the linearised vector to generate the final destination vector pCAM:PAT:GW (d). Promoters (green arrows), antibiotic resistance genes (blue arrows), polyA positions (red lines), T-DNA borders (black boxes), *att* sites (red boxes), *ccdB* and chloramphenicol resistance gene (*Cm^R*) shown. Relevant restriction enzyme recognition sites indicated. Figure provided by M. T. Page (University of Bath).

3.3.4 PCR amplification and cloning of target sequence into Gateway® donor vector

The coding region of *ASCORBATE PEROXIDASE*, *GALACTURONIC ACID REDUCTASE*, *γ-GLUTAMYL-CYSTEINE SYNTHETASE*, *CATALASE* and *SUPEROXIDE DISMUTASE* (Table 3.1), previously isolated and cloned from different plant host species, were PCR amplified (Section 2.1.1) using sequence specific primers (Tables 2.1 and 3.1).

Table 3.1 Target sequence used in expression constructs.

Target sequence	Amplification primers*	Accession number	Amplicon length (bp)	Origin	Reference
<i>ASCORBATE PEROXIDASE</i> (<i>MecAPX2</i>)	apxF1 & apxR1 antiapxF1 & antiapxR1	AY973622	775	<i>M. esculenta</i> (Cassava)	(Reilly <i>et al.</i> , 2007)
<i>CATALASE</i> (<i>MecCAT1</i>)	catF1 & catR1 anticatF1 & anticatR1	AF170272	1,507	<i>M. esculenta</i> (Cassava)	(Reilly <i>et al.</i> , 2001)
<i>GALACTURONIC ACID REDUCTASE</i> (<i>GalUR</i>)	garF1 & garR1 antigarF1 & antigarR1	AF039182	960	<i>Fragaria ananassa</i> (Strawberry)	(Agius <i>et al.</i> , 2003)
<i>γ-GLUTAMYL-CYSTEINE SYNTHETASE</i> (<i>AtGSH1</i>)	gshF2 & gshR2 antigshF1 & antigshR1	AF419576	1,569	<i>Arabidopsis thaliana</i> (Thale cress)	(May & Leaver, 1994), obtained from RIKEN, Japan
<i>SUPEROXIDE DISMUTASE</i> (<i>MecSOD2</i>)	sodF1 & sodR1 antisodF1 & antisodR1	AY642137	487	<i>M. esculenta</i> (Cassava)	(Reilly <i>et al.</i> , 2007)
StPAT (<i>PATATIN</i>) promoter	Pat-Pst F Pat-Pml R	Unpublished	1,020	<i>S. tuberosum</i> (Potato)	(Ihemere <i>et al.</i> , 2006)
<i>GUSPlus</i> reporter gene	GUSattfor GUSattrev	AF354045	2,078	<i>Saccharomyces</i>	(pCAMBIA, 2011)

* nucleotide sequence provided in Table 2.1

The *attB* sites required for the first stage of Gateway® cloning are 29 nucleotides in length, which is too long to integrate into a single primer and thus a two-step PCR was performed. The coding sequence amplification primers comprised approximately 10 nucleotides of *attB* site sequence immediately adjacent to the initiation codon. Also included in the 5' primer was the native Kozak sequence for each of the genes of interest to ensure maximal expression (Kozak, 1987; Tables 2.1 and 3.1). An aliquot of the first PCR was used as the template DNA for a further PCR (Section 2.1.1) using primers attB1 and attB2 (Table 2.1) to amplify the target sequence and incorporate the full-length *attB* recombination sites. To serve as a control for plasmid stability and monitor transformation progress, the coding sequence of the *GUSPlus* reporter gene in pCAMBIA 1305.1 was PCR amplified using primers GUSattfor and GUSattrev as described above (Tables 2.1 and 3.1). The PCR products were successfully cloned into an intermediate vector (pDONR™/Zeo; Invitrogen) using BP clonase™ as outlined by the manufacturer (Invitrogen). pDONR™/Zeo was selected because it contains the Zeocin™ resistance gene (*zeo*) rather than the more conventional *nptII*. pCAM:PAT:GW contains *nptII* for bacterial selection and thus utilising a donor vector with the same antibiotic resistance gene would interfere with screening of colonies in subsequent stages of cloning. The products of the BP clonase™ catalysed reaction were used to transform One Shot® Omnimax 2-T1 Chemically Competent *E. coli*, which were selected on LB agar media supplemented with 50 µg ml⁻¹ Zeocin™ (Section 2.2.2). Extracted plasmid DNA (Section 2.2.6) was restriction enzyme digested using *BsrGI* to screen for colonies with the expected banding pattern. Selected samples were sequenced using vector primers (M13F & M13R; Table 2.1) to confirm integrity of the target sequence. This procedure resulted in the successful creation of intermediate (or entry) clones named pENTR™-*MecAPX2*, pENTR™-*MecCAT1*, pENTR™-*GalUR*, pENTR™-*AtGSH1*, pENTR™-*MecSOD2* and pENTR™-*GUSPlus*.

Selected pENTR™-based clones were recombined with the pCAM:PAT:GW cassette using LR clonase™, as outlined by the manufacturer (Invitrogen). The plasmid mix was used to transform One Shot® *ccdB* Survival™ T1R Chemically Competent *E. coli*, plated on LB agar media supplemented with 50 µg ml⁻¹ kanamycin. Plasmid DNA was extracted from bacterial colonies and sequenced with primers DESTSeqF1 and DESTSeqR1 (Section 2.4.9) to confirm the successful integration of target sequence. The final expression constructs were termed pDEST™-*MecAPX2*, pDEST™-*MecCAT1*,

pDEST™-GalUR, pDEST™-AtGSH1, pDEST™-MecSOD2 and pDEST™-GUSPlus (Figure 3.6).

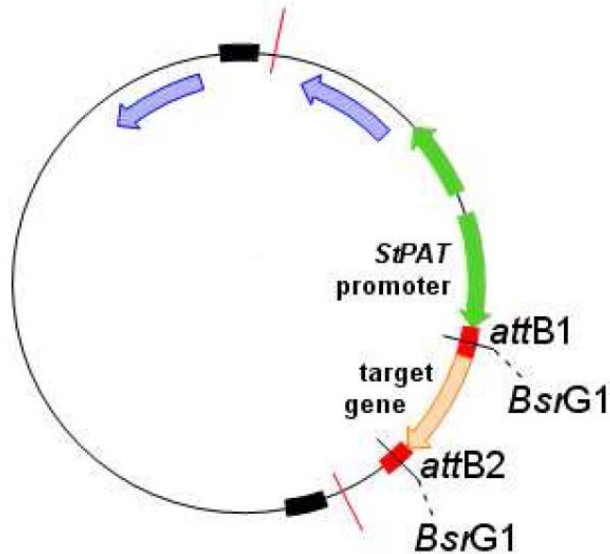


Figure 3.6 Simplified map of the pCAM:PAT:GW expression cassette. Promoters (green arrows), antibiotic resistance genes (blue arrows), positions of PolyA (red lines) and the T-DNA borders (black boxes) shown. Relevant restriction enzyme recognition sites indicated. Figure provided by M. T. Page (University of Bath).

3.3.5 PCR amplification and cloning of target sequence in antisense orientation and negative control

Antisense constructs of the selected genes were generated to assess to what extent the over-expression constructs are regulated and influenced by the *StPAT* promoter and ROS pathways. The silencing of genes involved in modulation of ROS would in theory lead to increased oxidative stress. Although it is also possible that endogenous genes would compensate for altered gene expression. The approach to clone the genes in antisense orientation was fundamentally the same as for the over-expression/sense orientation constructs described in the sections above. The important difference is that the *attB1* sequence was incorporated in the complementary-strand rather than the sense-strand primer. Likewise, the sense-strand primers were designed with the *attB2* sequence (Table 2.1). The swapping of the *attB* sites at the ends of the PCR-amplified coding sequences allows the gene to be orientated in the vector so that it is transcribed

in the antisense. The generated clones were termed pDEST™-antiAPX, pDEST™-antiCAT, pDEST™-antiGalUR, pDEST™-antiGSH1 and pDEST™-antiSOD. Lastly, the pDEST™-*GUSPlus* construct was digested using restriction enzymes *Pst*I and *Nco*I (Section 2.4.4) to remove the *St*PAT promoter. The linearised vector was blunt-ended (Section 2.4.5), re-ligated and used to transform *E. coli* One Shot® TOP10 cells. Cells were plated on LB agar media supplemented with 50 µg ml⁻¹ kanamycin and colonies were subsequently screened following plasmid extraction *via* sequencing using primers CAMBSeqF1 and CAMBSeqR1, which anneals to the *GUSPlus* sequence (Table 2.1; Sections 2.2.6 and 2.4.9) to ensure the expected promoterless construct (pDEST™-*GUSPlus*(-)*PAT*) had been created (Figure 3.7). Transformation using a promoterless construct should offer insights into the function and regulation of expression of *St*PAT *in planta*.

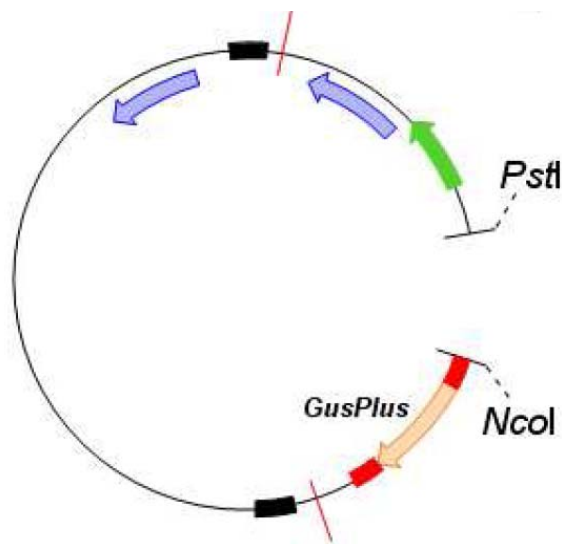


Figure 3.7 Creation of pDEST™-*GUSPlus*(-)*PAT*. pDEST™-*GUSPlus* was digested with restriction enzymes *Pst*I and *Nco*I (recognition sites located immediately upstream and downstream, respectively, of the *St*PAT promoter). The linearised vector was re-ligated to generate a promoterless construct. Antibiotic resistance genes (blue arrows), *CaMV35S* promoter (green arrow), polyA positions (red lines), T-DNA borders (black boxes), *att* sites (red boxes), *GUSPlus* and positions of selected restriction enzyme sites shown. Figure provided by M. T. Page (University of Bath).

3.4 DISCUSSION

This chapter describes the successful modification of the pCAMBIA 1305.1 vector to incorporate the root-specific *St*PAT promoter and components for Gateway® cloning of the target genes. The basic cassette, named pCAM:PAT:GW, was used to clone the coding regions of *MecAPX2*, *MecCAT1*, *GalUR*, *AtGSH1*, *MecSOD2* and also *GUSPlus* (Table 3.1). The over-expression constructs are termed pDEST™-*MecAPX2*, pDEST™-*MecCAT1*, pDEST™-*GalUR*, pDEST™-*AtGSH1*, pDEST™-*MecSOD2* and pDEST™-*GUSPlus* and the corresponding antisense constructs pDEST™-antiAPX, pDEST™-antiCAT, pDEST™-antiGalUR, pDEST™-antiGSH1, pDEST™-antiSOD were also successfully developed to serve as a direct comparison to their sense-orientated constructs. The *GUSPlus* reporter gene construct (pDEST™-*GUSPlus*) will serve as a visual control during the transformation process (Chapter 4) and ultimately offers a means to observe gene expression in plant tissue. Although GUS assays result in destruction of the tissue sample, it is a superior marker system compared to *luc* or *gfp* where experience and specialist equipment is required. The over-expression and antisense constructs listed above were used to successfully electroporate *Agrobacterium* strain LBA4404 (Sections 2.2.4 and 2.2.5) required for cassava transformation (Chapter 4). The strain of *Agrobacterium* has proven to influence transformation success (Li *et al.*, 1996) but LBA4404 has been used repeatedly in genetic transformation of cassava by various research groups (Ihemere *et al.*, 2006; Jørgensen *et al.*, 2005; Li *et al.*, 1996; Vanderschuren *et al.*, 2007; Vanderschuren *et al.*, 2009; Zhang & Gruissem, 2004). Attempts were made originally to convert the pCAM:PAT:GW into an RNAi cassette *via* the incorporation of components of the pHELLSGATE RNAi Gateway® vector (Helliwell & Waterhouse, 2005), but due to time constraints this proposal was not pursued. Importantly, the introduction of the Gateway® system in pCAM:PAT:GW allowed efficient cloning of the genes without the potential complications of restriction enzyme site digestion and ligation. This unique cassette has now been adopted by researchers at the University of Bath to generate transgenic cassava (K. A. Jones, *pers. comm.*), as well as by scientists at ETH Zürich, Switzerland, for research into improved starch and biofuel production (S. C. Zeeman, *pers. comm.*).

4 TISSUE CULTURE AND TRANSFORMATION OF CASSAVA

4.1 INTRODUCTION

4.1.1 CASSAVA BIOTECHNOLOGY

4.1.1.1 *in vitro* tissue culture of cassava

The unique capacity of plants to reproduce from vegetative/non-zygotic tissue (*via* somatic embryogenesis) has been exploited for cassava tissue culture. Somatic embryos have been generated from mature seed, leaf explants (Stamp & Henshaw, 1982), shoot apical or axillary meristem (i.e. bud) tissue (Szabados *et al.*, 1987) cultured on MS medium supplemented with auxin (e.g. the synthetic auxins 2,4-dichlorophenoxyacetic acid (2,4-D) or picloram) and/or cytokinin (e.g. BAP). Division of morphogenetically competent cells occurs in the adaxial tissues leading to the development of embryogenetic protrusions (torpedo structures) and subsequently primary somatic embryos (Stamp, 1987; Figure 4.1a). Maintenance and further propagation of these embryos on MS media supplemented with auxin prevents plant development and gives rise to successive cycles of secondary somatic embryos. The transition to continuous embryogenic gene expression rather than germination has not been investigated in cassava but DNA methylation influenced by auxins is thought to be a contributory factor (George *et al.*, 2008b). Cycling of secondary somatic embryos is not indefinite since the tissue is likely to mature and the process of somatic embryogenesis will need to be reinitiated to replenish *in vitro* stocks. Somatic embryos cultured on basic MS media incubated with a 16 hr photoperiod leads to the development of cotyledons and will eventually regenerate into plantlets (Stamp & Henshaw, 1986; Stamp & Henshaw, 1987). The inclusion of a cytokinin (e.g. BAP) in the media can be used to induce shoot development directly from non-differentiated tissue (i.e. avoiding embryo germination), a process referred to as shoot organogenesis (Figure 4.2). Importantly, the maturation/regeneration of somatic embryos not only tests the viability of embryos to germinate, but also serves as a conduit in the production of pathogen-free plantlets for distribution to researchers, breeders and farmers (Raemakers *et al.*, 1999).



Figure 4.1 Somatic embryogenesis in cassava cultivar TMS60444. Primary somatic embryos from axillary meristematic tissue (i.e. buds) **(a)**, clusters of FEC (indicated by red arrows) emerging on secondary somatic embryos **(b)**, maturing embryo from FEC, root axis and emerging cotyledons visible **(c)**.

Secondary somatic embryos can also be cultured on GD media supplemented with picloram to establish friable embryogenic callus (FEC; Figures 4.1b and 4.2). FEC contain hundreds/thousands of morphogenetically competent, totipotent cells approximately ≤ 1 mm in diameter that rapidly proliferate in a disorganised manner - a process enhanced by transferring the developing FEC to SH liquid medium containing picloram (Raemakers *et al.*, 2006; Taylor *et al.*, 1996). From FEC, embryos can mature on MS-based media (lacking auxin but containing cytokinin; Figure 4.1c) and eventually regenerate into plantlets, completing the cycle of somatic embryogenesis and cassava regeneration (Figure 4.2). Protoplasts, which serve as relatively amenable systems for biochemical analyses and transformation, have been isolated from cassava leaf tissue (Shahin & Shepard, 1980) and FEC (Sofiari *et al.*, 1998). However protoplast isolation further complicates the tissue culture process and became largely redundant for modification of cassava following successful transformation of FEC - an ideal host tissue for maximising the probability of integration of T-DNA into large numbers of cells. The efficiency by which FEC can be produced is cultivar dependent and research today almost exclusively uses TMS60444, developed at IITA in Nigeria. Notwithstanding, various publications report successful generation of FEC and/or secondary somatic embryos from other cultivars, including Bujá Petra and Rosinha from South America (Ibrahim *et al.*, 2008), Adira 4 from Indonesia (Schreuder *et al.*, 2001) and various TME and TMS lines from Africa (Hankoua *et al.*, 2005).

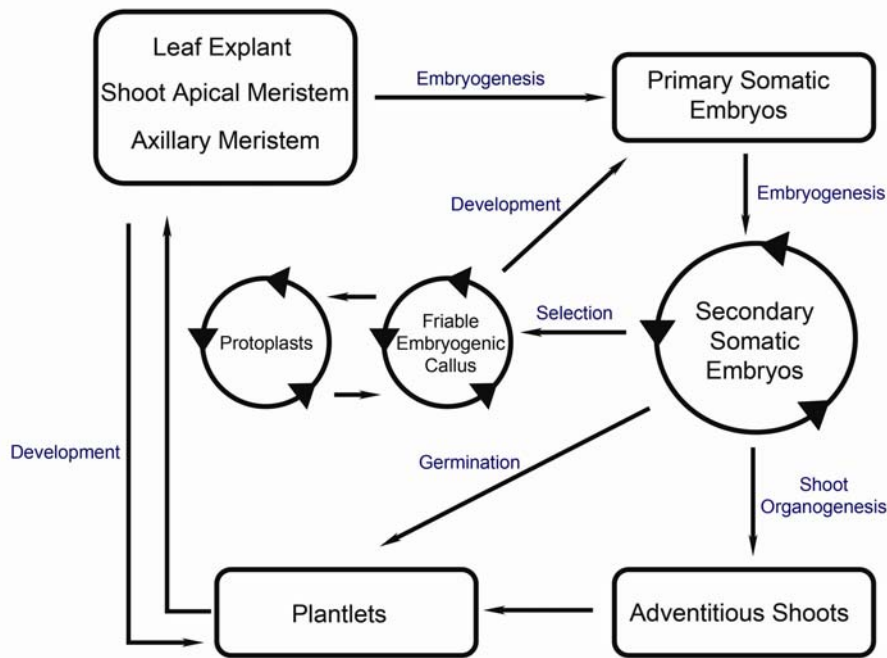


Figure 4.2 Schematic representation of somatic embryogenesis and regeneration of cassava.

4.1.1.2 Cassava transformation techniques

Transformation of cassava evolved in the 1990s with the implementation of electroporation, biolistic and *Agrobacterium*-mediated techniques. Initially only transient gene expression was obtained in embryogenic tissues electroporated with a plasmid containing *uidA* (Luong *et al.*, 1995), as well as in biolistically inoculated leaf (Franche *et al.*, 1991) and root tissue (Arias-Garzon & Sayre, 1993). However, Li *et al.* (1996) used *Agrobacterium*-mediated transformation of somatic embryo cotyledons (also using *uidA*-containing plasmids) and generated stably transformed material *via* shoot organogenesis. In the same year, transgenic plantlets were also regenerated from FEC transformed with pUC19 (containing *nptII* and *uidA*) by biolistic inoculation (Schöpke *et al.*, 1996; Taylor *et al.*, 1996). Biolistic bombardment of somatic cotyledons has since also resulted in stably transformed cassava (Zhang *et al.*, 2000a). Unfortunately, various difficulties were encountered with all these techniques, including poor regeneration efficiency of plantlets from somatic embryos, intrinsic variation (including tissue quality) between transformation experiments (Schreuder *et al.*, 2001), difficulty in using the protocol with farmer-preferred cultivars and the potential for chimeras and somaclonal variation (Raemakers *et al.*, 1997; Raemakers *et al.*, 2001). Eventually, *Agrobacterium*-

mediated transformation of FEC (González *et al.*, 1998; Zhang *et al.*, 2000b) became more widely adopted since it was regarded to be more efficient, less expensive, increases the likelihood of single-insert lines and carries less risk of generating chimeras since differentiated tissue is not used. However, the technique remained complicated, laborious and uptake by research groups proved limited.

Peer-reviewed papers exploiting transgenic cassava are seldom published and usually report only a few independent transgenic lines. For example, Chellappan *et al.* (2004) reports 5-13 lines of cassava that confer resistance to geminiviruses. Only three lines of ACMV-resistant plants were generated by Vanderschuren *et al.* (2007) using *Agrobacterium*-mediated transformation of FEC. Similarly, Ihemere *et al.* (2006) screened 872 embryo explants identifying only five lines with enhanced starch production following *Agrobacterium*-mediated transformation of somatic cotyledons. The same approach resulted in a 1% success rate (equivalent to a single plant line) in generating transgenic material by Sarria *et al.* (2000). Jørgensen *et al.* (2005), however, was more successful obtaining in excess of 80 independent lines using *Agrobacterium*-mediated transformation of somatic cotyledons to generate cassava with reduced cyanogenic glucoside content, but tissue preparation and screening was considerably laborious (K. Jørgensen, *pers. comm.*). Evidently, whilst cassava transformation has been implemented by several research groups, there remains significant variation in success that likely reflect inherent problems with the different protocols and acquisition of knowledge.

4.2 RESEARCH OBJECTIVES

The primary objective was to generate transgenic cassava *via Agrobacterium*-mediated transformation of FEC using the constructs described in Chapter 3. However, numerous constraints and problems frustrated the achievement of this objective, so much so that for continuation of the project these problems needed to be surmounted *via* a comprehensive review of the protocol(s). This chapter briefly documents the preliminary data that led to the appraisal, followed by the key experiments undertaken to troubleshoot the protocol and produce transgenic cassava. The experiments are presented in the following format: Observations/Background, Experiment Outline, Results. The collective data and results are discussed.

4.3 RESULTS

4.3.1 Transformation of cassava utilising the published protocol

Cassava tissue culture and transformation were undertaken as described (Zhang & Gruissem, 2004; Zhang & Puonti-Kaerlas, 2004), summarised in Section 2.5 and with guidance by P. Zhang (Shanghai Institutes for Biological Sciences, China). Using this method, 14 independent batches of FEC (cultivar TMS60444) were produced and used for *Agrobacterium*-mediated transformation with the designed expression constructs (Table 3.1), including pCAMBIA 1305.1 and pDEST™-GUSPlus, which contain the GUSPlus reporter gene to visualise transformation progress. A total of 141 transformation experiments (i.e. co-cultivation of *Agrobacterium* and several plates of FEC per expression construct) were attempted and 155 *in vitro* plantlets were generated (Table 4.1). However, none of the plantlets were transgenic as evidenced by rooting assays (Section 2.5.2), where transferred apical growth tips failed to develop roots in CBM+H40 (CBM supplemented with 40 µg ml⁻¹ hygromycin). Furthermore, no products were observed in agarose gels (Section 2.4.2) following PCR-amplification of *hptII* and relevant transgene using specific primers (Table 2.1; Section 2.1.2) from isolated DNA (Section 2.3.1; data not shown). In brief, all attempts to generate transgenic plantlets failed. The stability or possible detrimental influence of gene expression or the cassette *per se* upon transformation and regeneration success were eliminated following successful transformation of *Arabidopsis* (Page, 2009).

Table 4.1 Transformation of independent batches of FEC using expression constructs.

Gene orientation	Expression construct	Number of FEC batches used [†]	Number of transformations* [§]	Number of <i>in vitro</i> plantlets
Over-expression (sense orientation)	pCAMBIA 1305.1	8	21	6
	pDEST™-GUSPlus	5	7	16
	pDEST™-AtGSH1	13	22	2
	pDEST™-GalUR	9	12	23
	pDEST™-MecAPX2	13	20	24
	pDEST™-MecSOD2	14	22	29
	pDEST™-MecCAT1	11	16	19
Antisense	pDEST™-antiGSH1	4	5	5
	pDEST™-antiGalUR	4	5	7
	pDEST™-antiAPX	3	3	7
	pDEST™-antiSOD	4	4	10
	pDEST™-antiCAT	4	4	7
TOTAL:		14	141	155

- † independent batches of FEC generated and maintained from cultivar TMS60444
- * number of occasions a stock of *Agrobacterium* harbouring an expression construct was cultured and used to transform FEC
- § tissue culture and transformation protocol based on Zhang & Gruissem (2004), Zhang & Puonti-Kaerlas (2004) and advice (P. Zhang)

Throughout the course of the investigation when the original protocol was used (approximately one to two years) numerous alterations and recommendations were tested (Table 4.2). These qualitative experiments served to improve knowledge and catalogue all aspects of the cassava transformation protocol, spawning more structured experiments to tackle identified bottlenecks. These experiments are outlined and discussed in detail in the following sections. It is noteworthy that the various difficulties encountered were not confined to this investigation but were also experienced by other researchers in the group, highlighting the apparent lack of a robust and reliable protocol.

Table 4.2 Preliminary experiments and considerations to improve the *Agrobacterium*-mediated cassava transformation protocol.

Question	Observations & considerations	Experiment outline & conclusions
Is stringent autoclaving of media affecting pH and other properties?	Autoclaving is known to affect properties of media and therefore it was wondered if FEC development, in particular, was being compromised by sub-optimal media. For example, discolouration of autoclaved media due to sucrose hydrolysis was frequently observed.	GD (pH 5.8) was prepared and the pH measured pre- and post-autoclaving. The pH dropped by approximately 0.3 following autoclaving and therefore the pH of pre-autoclaved media was adjusted to accommodate this fluctuation. Changes in pH were also closely associated with the addition of supplementary stock solutions sometimes added after autoclaving (see below). Additionally, CBM in particular was prone to sucrose hydrolysis and therefore a less stringent autoclave cycle was programmed to prevent this reaction whilst maintaining sterile conditions.
Is the preparation and storage of media and chemicals as advised by the manufacturer and transformation protocol?	Stocks of chemicals used for media preparation were poorly managed and being prepared slightly differently by researchers.	A comprehensive review into the preparation, storage and management of communal stocks was undertaken and led to the implementation of numerous standard operating procedures (SOPs). The SOPs were vital to ensure minimal variation between stocks and thus batches of media.
Are auxins and cytokinins (e.g. picloram and BAP) being decomposed during autoclaving?	Plant hormones used in culture media may not be heat labile and therefore their addition prior to autoclaving may be affecting media properties.	FEC (FEC4) were propagated on GD plates prepared with picloram added prior to or after media sterilisation. After three weeks incubation (28°C, 16 hr photoperiod) growth was visually assessed but no distinguishable difference was observed. Due to the increased risk of contamination and changes to media <i>via</i> addition of hormones post-autoclaving, current practise was continued whilst acknowledging that some degradation of hormones probably occurs.
Are the protocols outlined in Zhang & Gruissem (2004) resulting in sub-optimal media?	Due to the various considerations outlined above, it was questioned whether the adopted protocol was resulting in media of variable quality. Protocols were therefore obtained from the Donald Danforth Plant Science Centre (DDPSC; N. Taylor, <i>pers. comm.</i>) for comparison.	GD and MS-based media were prepared following DDPSC protocols. The preparation was largely similar to those outlined by Zhang & Gruissem (2004) but nutrients were added separately and supplements (e.g. hormones) were added after sterilisation. FEC propagated on GD plates developed as expected but the development of somatic embryos from leaf lobes on MS-based media was poor. However, this was attributed to the fact that leaf lobes were used rather than the media itself. Notably, the use of Noble agar at DDPSC resulted in a far superior media and was investigated further. Overall, the DDPSC media preparation protocols were probably more precise but they were also more labour-intensive and considered unnecessary. Please see Section 4.3.2 for further details.

Does the isolation and transfer of transformed FEC to fresh media improve regeneration capacity?	The protocol used at DDPSC states that it is necessary to hand-pick transformed FEC and transfer frequently to fresh media, thus isolating them from decomposing (non-transformed) FEC and constantly replenish nutrient availability.	<p>The following experiment was undertaken:</p> <ul style="list-style-type: none"> a) Propagation of FEC in SH liquid media b) Co-cultivation of <i>Agrobacterium</i> and FEC as described by Zhang & Gruissem (2004) c) Wash FEC in SH+C500 d) FEC in SH+C500+H15 (10 d) e) Transfer to MSN+C500+H25 and incubate for 2 weeks (28°C, 16 hr photoperiod). f) Select swollen, yellow FEC, transfer to fresh media and incubate (as above) for 2 weeks. <p>Unfortunately, FEC failed to regenerate and were discarded after one month.</p>
Was the concentration of hygromycin antibiotic hindering regeneration in the above experiment?	Hygromycin is known to negatively affect regeneration capacity of cassava embryos. Therefore the above experiment was repeated but using a reduced concentration of hygromycin antibiotic, as well as an increased period of incubation on MSN to further aid regeneration.	<p>The following experiment was undertaken:</p> <ul style="list-style-type: none"> a) Propagation of FEC in SH liquid media b) Co-cultivation of <i>Agrobacterium</i> and FEC as described by Zhang & Gruissem (2004) c) Wash FEC in SH+C500 d) FEC in SH+C500+H15 (10 d) e) Transfer to MSN+C500+H20 and incubate for 2 weeks (28°C, 16 hr photoperiod). f) Select swollen, yellow FEC, transfer to fresh media and incubate (as above) for 2 weeks. Repeat selection twice. <p>Unfortunately, FEC failed to regenerate and were discarded after two months cycled on MSN+C500+H20. Please see Section 4.3.4 for further details.</p>
Is the co-cultivation of FEC in SH liquid media and <i>Agrobacterium</i> affecting transformation capacity?	It appeared that FEC cultured in SH liquid media were becoming more globular than those on GD plates. Also, co-cultivation was currently performed for 4 d compared to the 30	<p>The following experiment was undertaken:</p> <ul style="list-style-type: none"> a) Propagation of FEC on GD plates b) Co-cultivation following DDPSC protocol – FEC transferred to a 6-well Petri dish, homogenised and 2 ml <i>Agrobacterium</i> suspension added and incubated for

	min incubation used at the DDPSC, where liquid media is avoided.	<p>30 min.</p> <p>c) Wash FEC in GD+C500</p> <p>d) Transfer to MSN+C500+H25 and incubate for 2 weeks (28°C, 16 hr photoperiod).</p> <p>e) Select swollen, yellow FEC, transfer to fresh media and incubate (as above) for 2 weeks.</p> <p>FEC failed to regenerate and were discarded after one month. Please see Section 4.3.3 for further details.</p>
Is co-cultivation of FEC using the DDPSC method more efficient than that currently used? Is subsequent regeneration being hindered by antibiotic concentration?	The co-cultivation protocol from the DDPSC seems less stressing for FEC and also avoids liquid media. However, no FEC regenerated in the above experiment suggesting post-transformation stages are a problem; possibly the concentration of antibiotic, which was reduced in an effort to promote growth.	<p>The following experiment was undertaken:</p> <p>a) Propagation of FEC on GD plates</p> <p>b) Co-cultivation following DDPSC protocol – FEC transferred to a 6-well Petri dish, homogenised and 2 ml <i>Agrobacterium</i> suspension added and incubated for 30 min.</p> <p>c) Wash FEC in GD+C500</p> <p>d) Transfer to MSN+C500+H10 and incubate for 2 weeks (28°C, 16 hr photoperiod).</p> <p>e) Select swollen, yellow FEC, transfer to fresh media and incubate (as above) for 2 weeks. Repeat selection twice.</p> <p>Some FEC regenerated into cotyledons but GUS assays revealed they were not transgenic, suggesting antibiotic selection or co-cultivation were ineffective. Please see Sections 4.3.2 and 4.3.4 for further details.</p>
Is it necessary to remove FEC from GD propagation plates for inoculation with <i>Agrobacterium</i> ?	Zhang & Gruissem (2004) describe using FEC from SH liquid media whilst DDPSC transfers FEC to Petri dishes. Each approach extends the time required for transformation and increases resources used. Is it not possible to inoculate directly to clusters of FEC on GD?	<p>The following experiment was undertaken:</p> <p>a) Propagation of FEC on GD plates</p> <p>b) Co-cultivation <i>via</i> pipetting of <i>Agrobacterium</i> onto FEC clusters and incubate for 4 d, as described by Zhang & Gruissem (2004).</p> <p>c) Wash FEC in SH+C500</p> <p>d) FEC in SH+C500+H15 (10 d)</p> <p>e) Transfer to MSN+C500+H25 and incubate for 4 weeks (28°C, 16 hr photoperiod).</p> <p>FEC failed to regenerate and were discarded after one month.</p>

With the exclusion of the SH liquid media stage prior to co-cultivation is it necessary to propagate FEC on SH plates instead to improve regeneration?	The SH liquid media is intended to mature the FEC thus improving capacity for transformation and regeneration. However, is this critically important? And does antibiotic concentration after co-cultivation negatively affect selective regeneration?	<p>The following experiment was undertaken:</p> <ul style="list-style-type: none"> a) Propagate FEC on SH plates b) Co-cultivation <i>via</i> pipetting of <i>Agrobacterium</i> onto FEC clusters and incubate for 4 d, as described by Zhang & Gruissem (2004). c) Wash FEC in SH+C500 d) Transfer to SH+C500+H5 and SH+C500+H20 plates and incubate for 2 weeks (28°C, 16 hr photoperiod). e) Transfer to MSN+C250+H20 <p>FEC failed to regenerate and were discarded after one month. Please see Section 4.3.3 for further details.</p>
Is it more appropriate to use GD-based solid media after co-cultivation rather than SH-based liquid media?	FEC cultured on SH media appear to be inefficiently transformed and incapable of regeneration, yet FEC on GD remain friable and propagate successfully. Additionally, it would seem logical to wash the FEC in liquid media containing the basal salts of the following incubation phase.	<p>The following experiment was undertaken:</p> <ul style="list-style-type: none"> a) Propagate FEC on GD plates b) Co-cultivation <i>via</i> pipetting of <i>Agrobacterium</i> onto FEC clusters and incubate for 4 d, as described by Zhang & Gruissem (2004). c) Wash in GD+C500 d) Transfer to GD+C250+H5 and also GD+C250+H20 and incubate for 2 weeks (28°C, 16 hr photoperiod). e) Transfer to MSN+C500+H20 <p>Some FEC from GD+C250+H5 regenerated to establish green cotyledons but not all were transgenic, suggesting poor transformation efficiency and/or sub-optimal concentrations of hygromycin. Please see Section 4.3.3 for further details.</p>
Is hygromycin hindering regeneration and should an alternative antibiotic selectable marker be used?	Hygromycin may compromise regeneration if protocol is not optimised. Recommendations were made to test the protocol using <i>nptII</i> containing plasmids (N. Taylor, <i>pers. comm.</i>).	Media preparation, tissue culture techniques and transformation using <i>Agrobacterium</i> harbouring pCAMBIA 2301 (an <i>nptII</i> , <i>uidA</i> plasmid and selected using paramomycin) was undertaken according to the protocol from DDPSC. Interestingly, whilst embryos did successfully regenerate to form cotyledons none were transgenic, suggesting recommended concentrations of paramomycin were insufficient.

4.3.2 EXPERIMENT I: to determine the extent that light, media setting agent and culture chamber affect FEC cultivation.

4.3.2.1 Experiment I: Observations/Background

The culturing of FEC (Section 2.5.1) invariably resulted in a number of complications. Firstly, the FEC appeared very moist, soft and white on the GD media rather than being friable and yellowish in colour (Figure 4.3a). Secondly, moisture collected on the lid of the culture dishes during incubation, which presumably affected light penetration as well as media properties (Figure 4.3b). Thirdly, sieving of FEC cultured in SH liquid media prior to transformation to remove non-embryogenic friable callus (NEFC) – rapidly growing, disorganised translucent callus consisting of large vacuolated cells in a liquid matrix (Taylor *et al.*, 1996) - unfortunately appeared to damage the tissue, resulting in globular FEC. Lastly, the SH liquid media culturing stages, both prior to and following co-cultivation with *Agrobacterium*, was highly susceptible to microbial contamination with losses habitually between 50-100%. Collectively these problems dramatically hindered the ability to transform FEC.

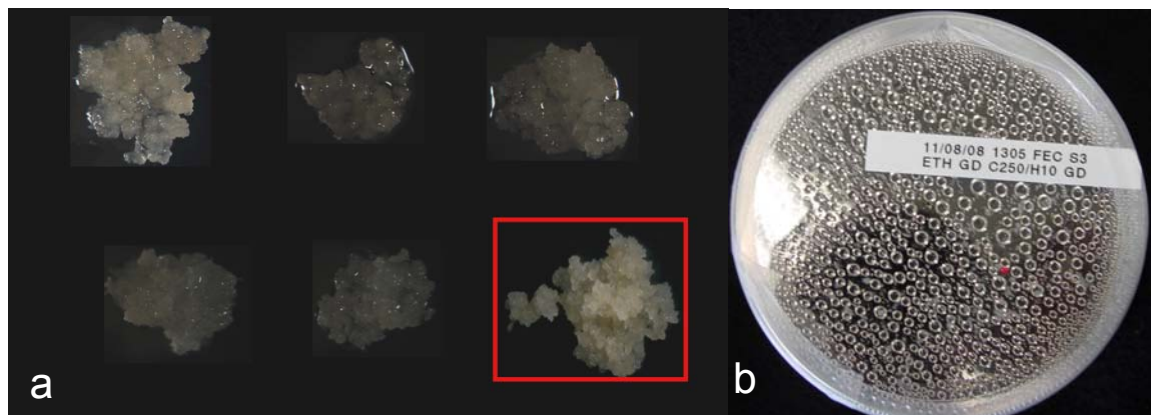


Figure 4.3 Morphological variation in FEC cultured on GD media. A selection of FEC clusters, which appear pale and moist compared with the ideal FEC (boxed in red) that is more friable and yellowish in appearance (a). Moisture accumulates on culture plates during incubation (b), which in-turn possibly affects FEC morphology.

4.3.2.2 Experiment I: Outline

Two independent batches of FEC (FEC7 and FEC9, generated one month apart) were sub-cultured on 48 GD plates (approximately eight clusters of FEC per plate); 24 plates were prepared with Gelrite™, as described in Zhang & Gruissem (2004; Section 2.5.1),

whilst the other 24 contained Noble agar, a highly purified setting agent (Difco™). 12 plates prepared with each setting agent were wrapped in aluminium foil (to simulate continuous darkness) whilst the other half remained uncovered and exposed to 16 hr photoperiod. The plates were incubated in three different chambers – an advanced, climate controlled incubator (Sanyo MLR Plant Growth Incubator), Climate Controlled Room and a General Plant Growth Chamber (Weiss Gallenkamp), the latter of which had been used previously for experiments (Table 4.1). Conditions in all chambers were set at 28°C with 16 hr photoperiod. Thus in total, two plates were prepared for each of the 24 different growth conditions, with each plate containing approximately eight clusters of FEC. Photographs of plates from each of the conditions, as well as close-up images of selected clusters of FEC (two per condition), were taken prior to incubation and after three weeks to assess FEC growth, morphology and the moisture content on culture dish lids. Following incubation, the FEC were co-cultivated for two d with *Agrobacterium* harbouring pCAMBIA 1305.1 (Section 2.5.2). Samples were used in a GUS assay to determine transformation efficiency (Figure 4.4).

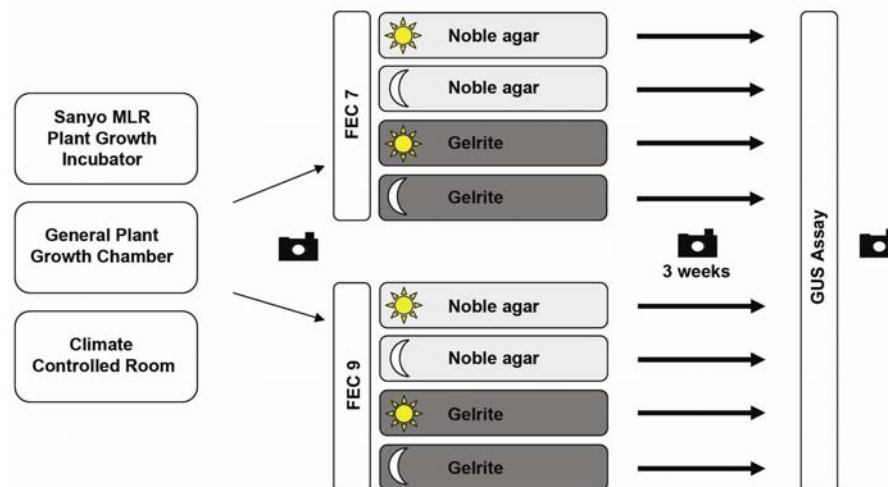


Figure 4.4 Procedure to assess FEC development under different growth conditions. FEC7 and FEC9 were sub-cultured on GD media prepared with Noble agar (light grey boxes) and Gelrite™ (dark grey boxes) and incubated in three different chambers in continuous darkness or 16 hr photoperiod (symbolised by a moon and sun, respectively). After three weeks the FEC were co-cultivated for 2 d with *Agrobacterium* harbouring pCAMBIA 1305.1 and then samples were used in a GUS assay. Two plates of FEC were prepared for each condition. Photographs were taken prior to and following incubation to assess FEC development, as well as following the GUS assay to determine the transformation capacity of FEC.

The production of primary somatic embryos from axillary meristem tissue (Section 2.5.1) was also suggested to affect FEC development. Leaf explants, which have been used successfully in tissue culture (N. Taylor, *pers. comm.*; Stamp & Henshaw, 1982), were therefore selected as an alternative source of meristematic tissue. Leaf lobes were isolated from 1-2 month old *in vitro* plantlets (cultivar TMS60444) and propagated on five CAM plates and incubated for 4 d at 28°C in darkness (Section 2.5.1). Bud tissue was also propagated on CAM and developing somatic embryos from both tissue types were photographed for comparative analysis.

4.3.2.3 Experiment I: Results

There was neither phenotypic abnormalities nor a discernible difference in the rate of growth of FEC7 and FEC9 maintained in light or dark conditions in all three climate chambers (data not shown). However, FEC9 appeared to have less NEFC and were more friable, reflecting the variation in quality between different batches of FEC tissue despite being produced *via* the same procedure. For simplicity, the results for FEC9 only will be presented below. FEC cultured on plates prepared with Noble agar were clearly more friable and yellowish in appearance following 22 d incubation compared with FEC cultured on Gelrite™-containing plates, irrespective of the climate chamber used (Figure 4.5). Overall, the blue/black precipitate indicative of successful transformation observed following the GUS assay (Figure 4.6) was most prevalent in FEC grown in the Sanyo MLR Plant Growth Incubator on Noble agar (Figure 4.6a). Interestingly, FEC that had been cultured on Gelrite™-containing GD media in the General Plant Growth Chamber failed to give a blue/black precipitate following the GUS assay (Figure 4.6f), suggesting either non-detectable levels of transformation or transformation had failed. Only culture plates incubated in the Sanyo MLR Plant Growth Incubator were consistently devoid of moisture on their lids, unlike those in the other two climate chambers tested (data not shown).

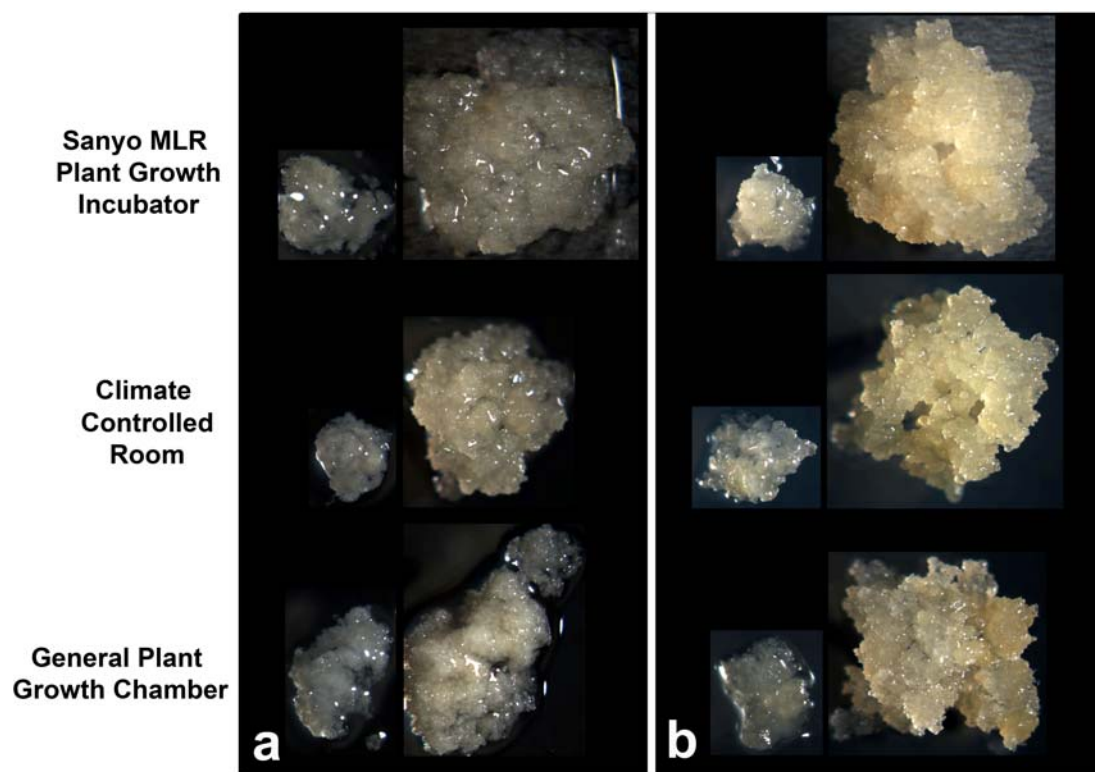


Figure 4.5 Development of FEC in different growth conditions. FEC9 sub-cultured on GD media prepared with Gelrite™ (**a**) or Noble agar (**b**). Clusters were photographed on 0 d (**left images**) and 22 d (**right images**) following incubation at 28°C, 16 hr photoperiod using climate chambers - Sanyo MLR Plant Growth Incubator, Climate Controlled Room and General Plant Growth Chamber.

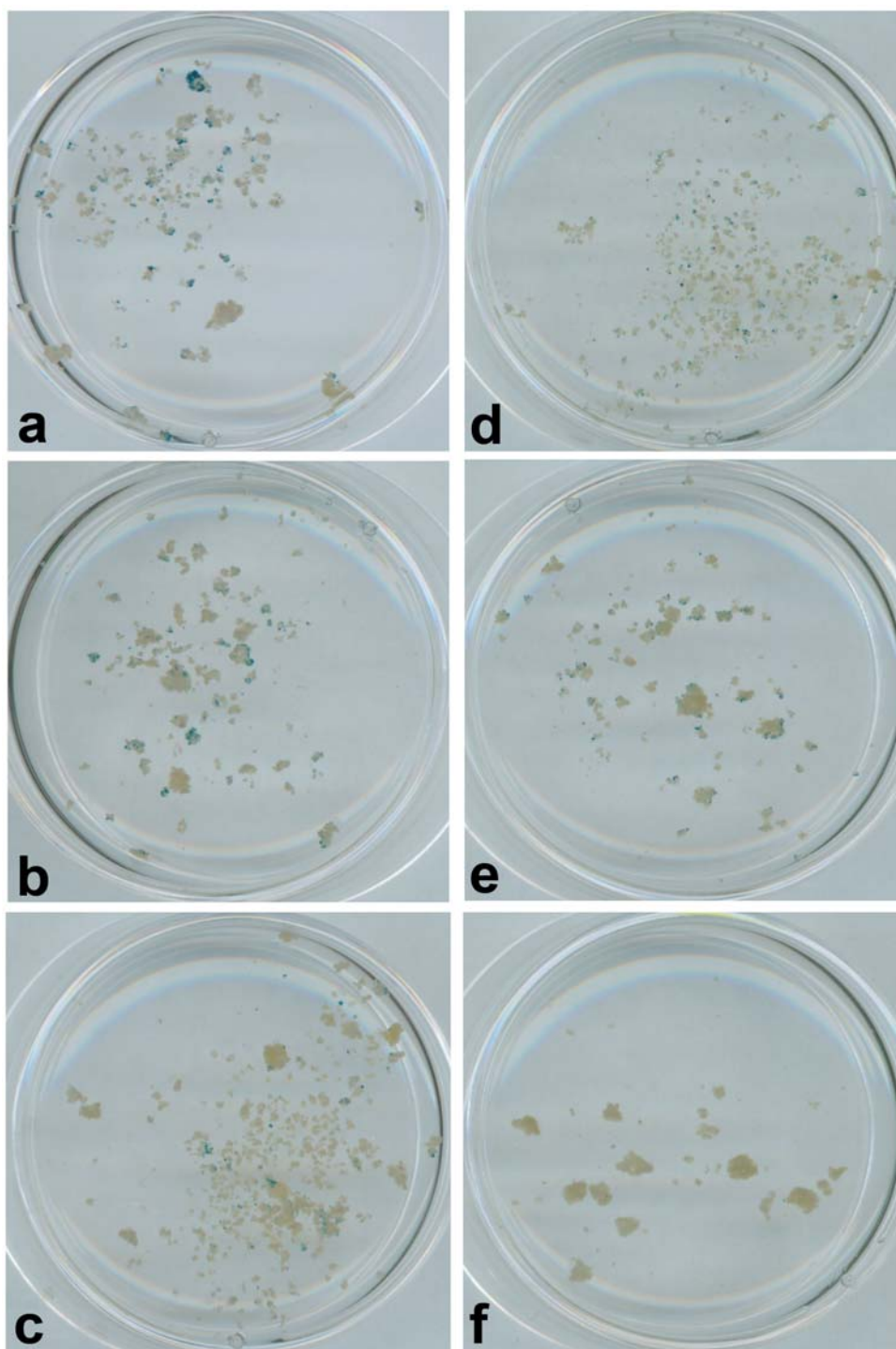


Figure 4.6 Results of GUS assay using FEC9 co-cultivated with *Agrobacterium* harbouring pCambia 1305.1 and cultured in different growth chambers. Blue/black precipitate can be observed in successfully transformed FEC cultivated on Noble-based media (**a**, **b** & **c**) and Gelrite™ (**d** & **e**) but not visible in FEC cultured on Gelrite™-containing GD and incubated in the General Plant Growth Chamber (**f**). Sanyo MLR Plant Growth Incubator (**a** & **d**), Climate Controlled Room (**b** & **e**) and General Plant Growth Chamber (**c** & **f**).

The experiment to assess somatic embryo and FEC development from leaf explants, as described by N. Taylor, proved to be a laborious process. Not only was the isolation of leaf lobes (Figure 4.7a) more fiddly than the removal of buds, but considerably more primary somatic embryos could be generated from bud tissue. Additionally, large quantities of NEFC was produced from leaf lobes (Figures 4.7b and 4.7c) compared with the use of axillary meristem tissue (observational data). The combination of time restrictions and these observations meant further investigation into the sub-culturing of secondary somatic embryos and the production of FEC was unwarranted. Initiation of all cassava tissue culture for this investigation therefore used axillary meristematic tissue.

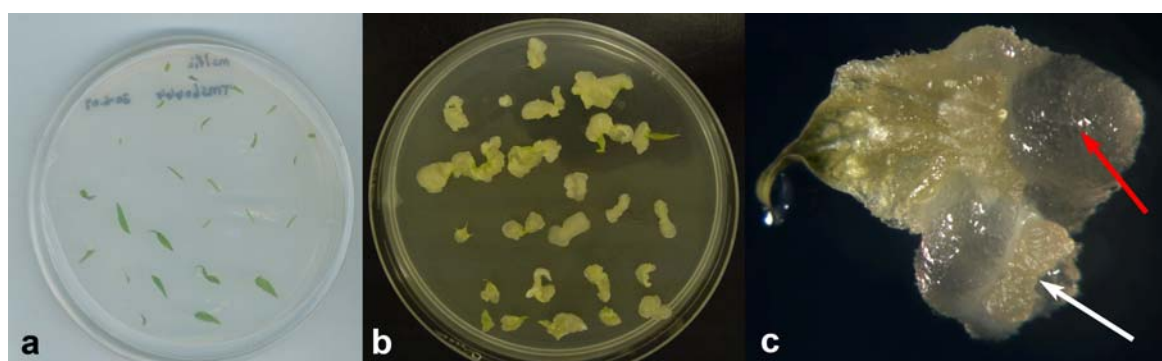


Figure 4.7 Primary somatic embryo development from leaf explants. Leaf lobes were isolated from *in vitro* TMS60444 plantlets and plated on CAM (a) that were incubated (28°C, continuous dark) for 4 d. Large quantities of NEFC (b) and indicated by red arrow in (c) were observed and few primary somatic embryos developed, indicated by white arrow in (c).

4.3.3 EXPERIMENT II: to determine whether FEC cultivation in SH liquid media negatively impacts on FEC morphology, transformation and regeneration.

4.3.3.1 Experiment II: Observations/Background

The cultivation of FEC in SH liquid media (Section 2.5.1), both prior to and following *Agrobacterium* co-cultivation (Figure 2.2), was time-consuming and highly susceptible to microbial contamination. Media needed to be replenished three times per week for a total of approximately five weeks and it was common to lose between 50-100% of the FEC tissue at these stages, thereby seriously hampering progress. Furthermore, the morphology of salvaged FEC changed dramatically from the small, friable clusters observed on GD plates (Figure 4.8a) to more globular structures during culturing in the

SH liquid media (Figure 4.8b). In-turn, there was a reduction in the efficiency of transformation, as determined by GUS assays, suggesting these globular units were not efficiently transformed. Only a faint blue precipitate was observed in some FEC (Figure 4.8c) and it is questionable whether the blue hue is in fact due to specific expression of *GUSPlus in vivo* or simply a collateral affect of transformation, tissue type, non-specific gene expression or possibly microscopy technique. Whatever the explanation, the FEC were neither morphologically as expected nor was the extent of precipitate following a GUS assay in-keeping with published results (Zhang & Puonti-Kaerlas, 2004).

The globular FEC observed during cultivation in SH liquid media also had poor capacity to develop primary somatic embryos and regenerate cotyledons. The swollen, yellowish (presumed transformed) FEC appeared to survive selection on MSN+H25 (Section 2.5.2) for up to six weeks, compared with the decaying white (presumed non-transformed) FEC also seen (Figure 4.8d). However, they consistently failed to regenerate. Only following the transfer of these structures to CMM (lacking antibiotic selection) and then subsequently to CEM, COM and CBM (Section 2.5.2) did approximately 40% (observational data) regenerate into plantlets (listed in Table 4.1). However, molecular and biochemical analyses revealed these plantlets were non-transgenic.

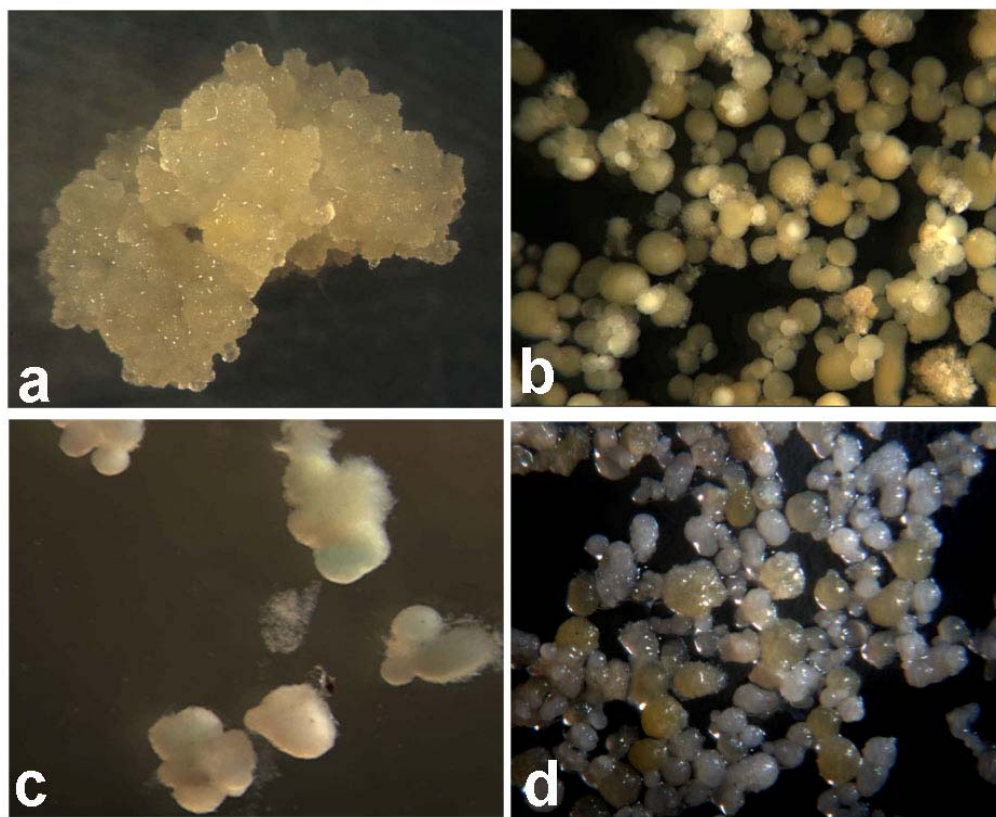


Figure 4.8 Morphological changes to FEC and impact on transformation and regeneration capacity. FEC sub-cultured on GD media containing Noble agar (**a**); in SH liquid media (**b**); GUS assay of FEC (from SH liquid media) following co-cultivation with *Agrobacterium* harbouring pCAMBIA 1305.1 (**c**); FEC plated on MSN+H25 - yellow FEC considered transformed and white FEC disintegrating (non-transformed) (**d**).

4.3.3.2 Experiment II: Outline

The culturing of FEC in SH liquid media introduced formidable problems to the protocol and was therefore omitted. Instead, co-cultivation of FEC and *Agrobacterium* would be tested directly on GD and SH plates, the latter serving as a substitute for SH liquid media. FEC9 were sub-cultured on approximately 10 Petri dishes of GD and SH media and incubated (28°C, 16 hr photoperiod) for 22 d. Photographs of selected clusters were taken immediately after sub-culturing and then again after 22 d to allow comparative phenotypic assessment of FEC development. After 22 d, *Agrobacterium* harbouring pCAMBIA 1305.1 (Section 2.2.5) was pipetted directly onto the FEC clusters, thus bypassing the SH liquid media cultivation used previously (Section 2.5.2). The plates were incubated for 4 d (16 hr photoperiod) at 24°C (as reported previously Section 2.5.2). The

co-cultivated material was scraped off using sterile forceps and washed in either GD or SH solution containing carbenicillin (500 mg L^{-1} ; GDS+C500 and SHS+C500, respectively) to suppress growth of *Agrobacterium*. This procedure was repeated until the wash solution was clear. FEC were then spread on either GD or SH plates containing carbenicillin (250 mg L^{-1} ; GD+C250 and SH+C250, respectively) for 2 d before transfer to media containing low concentrations of hygromycin (5 mg ml^{-1} ; GD+C250+H5 and SH+C250+H5) and incubated for 10 d (28°C , 16 hr photoperiod). Transformation success was assessed via a GUS assay immediately following co-cultivation and after 10 d incubation on the hygromycin containing media. The “acclimatisation” period following co-cultivation is broadly commensurate with the original protocol (Section 2.5) but instead was undertaken on plates rather than in liquid media.

4.3.3.3 Experiment II: Results

Wild-type (i.e. untransformed) FEC9 sub-cultured on GD and SH media and incubated for approximately 22 d yielded comparable quantities of FEC, although FEC on SH plates appeared more developed and yellowish in colour (Figure 4.9). Notably, this experiment was undertaken prior to the adoption of Noble agar (i.e. plates were prepared using Gelrite™) and thus the clusters appear moist (see Section 4.3.2 for further details).

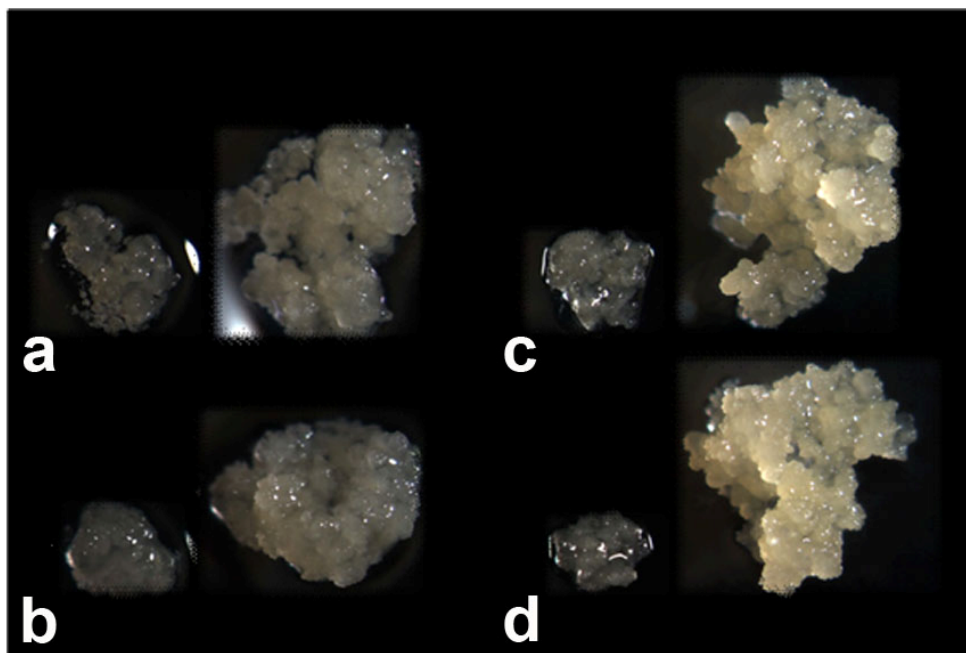


Figure 4.9 Examples of wild-type FEC sub-cultured on GD and SH media. FEC were plated on GD (a & b) and SH (c & d) media prepared with Gelrite™ and incubated (28°C , 16 hr photoperiod). Clusters were photographed immediately following sub-culturing (left images) and 22 d later (right images).

GUS assays of FEC co-cultivated with *Agrobacterium* harbouring pCAMBIA 1305.1 and subsequently incubated on SH+C250 and then SH+C250+H5 failed to produce a blue/black precipitate (data not shown). This indicates unsuccessful transformation of the SH cultured FEC material. Excitingly, however, FEC initially cultivated on GD+C250 revealed a significant increase in blue colouration following 10 d subsequent cultivation on GD+C250+H5 (Figure 4.10). This data suggests the acclimatisation period is crucial for efficient expression of *GUSPlus* and presumably the *hptII* antibiotic resistance gene.

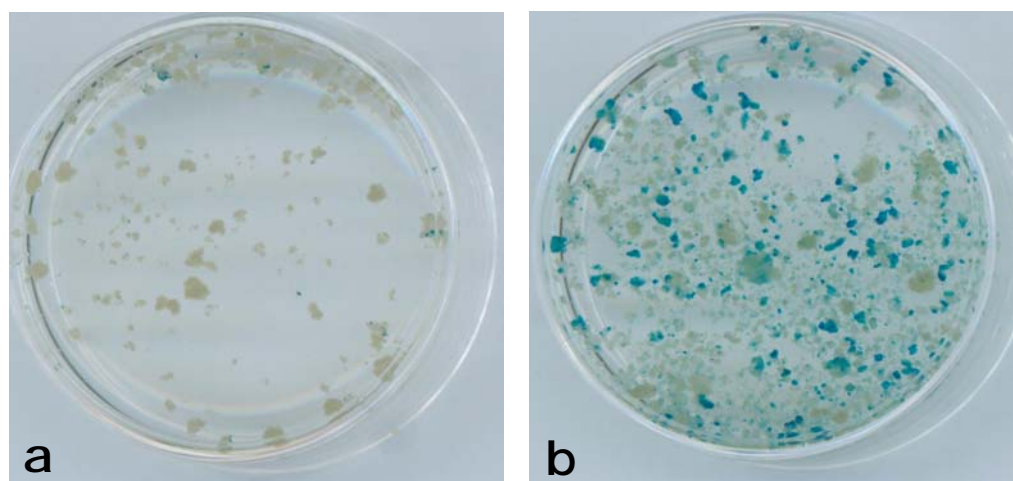


Figure 4.10 GUS assay using FEC co-cultivated with *Agrobacterium* harbouring pCAMBIA 1305.1 on GD plates. 2 d after co-cultivation and incubation on GD+C250 (a) and after 10 d subsequent incubation on GD+C250+H5 to further acclimatise FEC (b). Incubation at 28°C, 16 hr photoperiod.

4.3.4 EXPERIMENT III: to determine whether hygromycin hindered FEC regeneration and root development.

4.3.4.1 Experiment III: Observations/Background

The toxicity of hygromycin was hypothesised to be a credible problem due to two key observations. Firstly, as presented in Figure 4.8d, potentially transformed and non-transformed material on MSN+H25 is clearly distinguishable yet no FEC regenerated. Only when the material was placed on media devoid of hygromycin (i.e. CMM, CEM etc) did regeneration occur. Secondly, the recommended concentration of hygromycin in the rooting assay (40 mg L⁻¹; P. Zhang, *pers. comm.*) was even sufficient to hinder the

development of roots of confirmed hygromycin-resistant plants (positive controls) sourced from library stocks at ETH Zürich (data not shown). It was therefore necessary to investigate hygromycin toxicity and identify a range of concentrations suitable to allow regeneration of potentially transformed material, but sufficient to minimise development of non-transformed FEC. Interestingly, feedback from colleagues undertaking *Arabidopsis* transformation reported a wide variation in seed germination of transgenic material, which was accredited to the quality/purity of hygromycin being used and supplied from Duchefa.

4.3.4.2 Experiment III: Outline

Hygromycin toxicity was assessed for both root development and FEC regeneration. CBM was prepared with a range of hygromycin concentrations (0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.0, 1.6, 5, 7.5, 10, 12.5, 15, 17.5 and 20 mg L⁻¹, termed CBM+H0 → CBM+H20) using antibiotic from Roth AG, Switzerland. Two pots containing apical cuttings of wild-type TMS60444 plantlets and another two pots contained the apical meristems of known hygromycin-resistant plantlets (dsAC1-152 sourced from the ETH Zürich plant library), serving as the positive controls for each test concentration. In total, 90 wild-type and 90 *hptII* transgenic plantlets were used to assess 15 different concentrations of hygromycin in CBM. The material was incubated (28°C, 16 hr photoperiod) for three weeks and root growth photographed.

A range of hygromycin concentrations (0, 1.5, 2.5, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 20 mg L⁻¹) were also used to supplement MSN media (MSN+H0 → MSN+H20) to assess the impact upon FEC regeneration. Clusters of FEC9 were sub-cultured on MSN (three plates per concentration and three clusters per plate) and selected clusters were photographed at 1, 2, 3 and 5 weeks incubation (28°C, 16 hr photoperiod) to assess regeneration capacity. The experiment was repeated and data collated.

4.3.4.3 Experiment III: Results

After three weeks incubation the wild-type TMS60444 cuttings failed to develop roots in media containing ≥1.6-5 mg L⁻¹ hygromycin (CBM+H1.6 and CBM+H5; Figure 4.11b-c). Conversely, all hygromycin-resistant cuttings developed roots in CBM containing even the highest concentrations of hygromycin (CBM+H20), in-keeping with expectations (Figure 4.9e-h). This experiment was repeated to verify the observations. Thus, effective

screening of plantlets *via* a rooting assay can be achieved using only 5 mg L⁻¹ hygromycin, a concentration 8-fold less than was recommended.

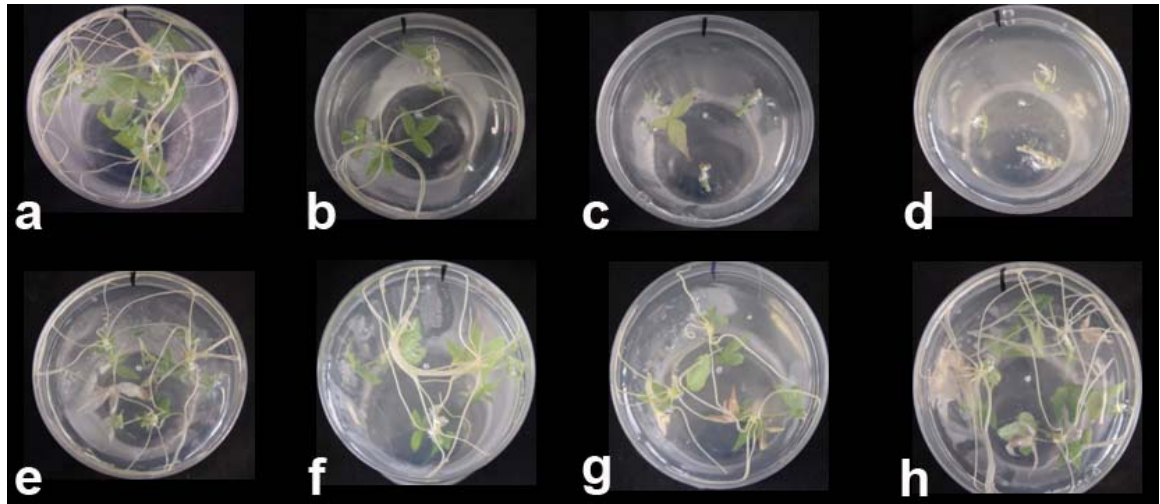


Figure 4.11 Rooting assay of wild-type TMS60444 and *hptII* transgenic cuttings in CBM supplemented with hygromycin. TMS60444 plantlets (a-d) and hygromycin-resistant transgenic material (e-h) in CBM+H0 (a & e); CBM+H1.6 (b); CBM+H5 (c & f); CBM+H10 (d & g) and CBM+H20 (h). Samples photographed after 3 weeks incubation (28°C, 16 hr photoperiod).

With regard FEC regeneration on MSN, there was a gradual decrease in the number of embryos/cotyledons developing on media containing increased concentrations of hygromycin (Figure 4.12). At 3 weeks incubation, FEC on MSN+H0 → MSN+H1.5 developed cotyledons (Figure 4.13) and after 5 weeks FEC on MSN+H15 were also regenerating, albeit few in number (average of 1.6). This suggests that FEC had either acquired resistance or, more likely, antibiotic in the media was degrading and possibly ineffective in reaching some of the uppermost FEC in the cluster. Notwithstanding, on MSN+H20 the hygromycin concentration was sufficient to suppress regeneration even at 5 weeks incubation, with the FEC appearing more callus-like in appearance and probably no-longer competent (Figure 4.13).

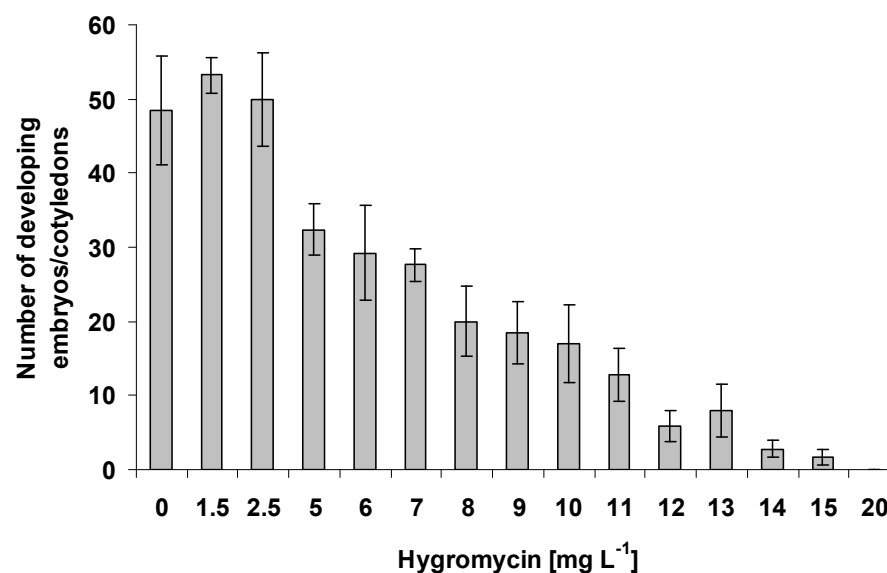


Figure 4.12 Number of developing embryos/cotyledons regenerating from wild-type TMS60444 FEC on MSN supplemented with hygromycin. Material was collected following 5 weeks incubation (28°C, 16 hr photoperiod). S.E. shown.

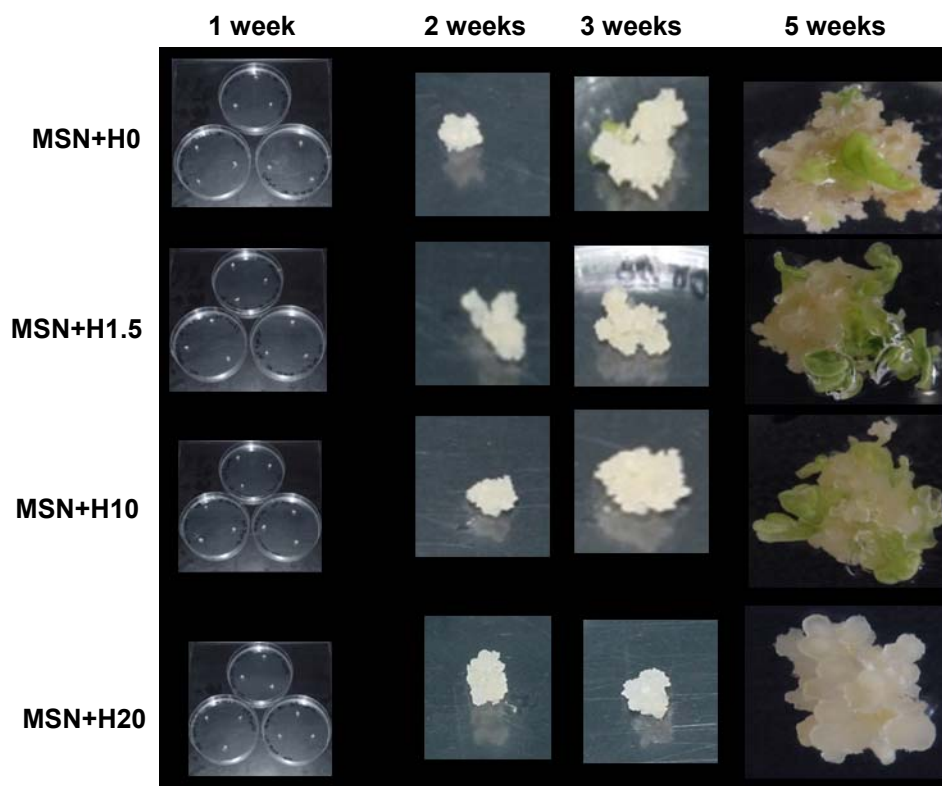


Figure 4.13 Wild-type TMS60444 FEC on MSN supplemented with hygromycin. Media prepared with hygromycin concentrations shown (MSN+H0 → MSN+H20). Material incubated (28°C, 16 hr photoperiod) and monitored over 5 weeks for embryo/cotyledon development.

4.4 DISCUSSION

The cassava transformation protocol as described by Zhang & Puonti-Kaerlas (2004) and Zhang & Gruissem (2004) resulted in complications, including variation in tissue quality, FEC morphology, poor regeneration capacity of embryos and microbial contamination. *Agrobacterium*-mediated transformation of FEC from cultivar TMS60444 using 12 expression constructs, including those that contain *GUSPlus* (Table 3.1), was attempted on 141 occasions from which 155 plantlets were regenerated. However, rooting experiments and molecular analyses revealed that none of these plantlets were stably transformed. Fundamental difficulties regarding cassava transformation were not confined to this project but also experienced by other members of the group at ETH Zürich (H. Vanderschuren, *pers. comm.*; J. Owiti, *pers. comm.*; M. Stupak, *pers. comm.*; C. Faso, *pers. comm.*). The consequence was that only a few stably transformed lines had been generated over a period of several years, despite daily and on-going experiments. The requisite for a reliable protocol for both this project and for cassava research generally evolved into an extensive re-evaluation of the procedure. In summary, between January 2007 and December 2008, all attempts at transformation failed comprehensively to deliver any transgenic plantlets. In approximately six months when the modified protocol was being implemented (early 2009), several hundred transgenic plants were produced. Moreover, due to on-going modifications and transformation attempts, approximately 50% of up-coming material was discarded due to constraints on space, resources, sanity(!) and crucially, provisional screening of generated plantlets revealed it was simply not required. The successfully modified protocol for *Agrobacterium*-mediated transformation of cassava FEC was recently published (Bull *et al.*, 2009).

4.4.1 FEC propagation and optimisation of growth conditions

The maintenance and propagation of FEC is fundamental for successful and efficient transformation. Although this process does require some skill and experience it is not the panacea to cassava tissue culture since the choice of climate chamber and media setting agent also affect FEC growth. Gelrite™, used by Zhang & Gruissem (2004), is a gellan gum derived from *Pseudomonas elodea* that provides a clear media, is used at lower concentrations and is relatively inexpensive compared to high-grade agars. However, some plant material is prone to hyperhydricity (particularly herbaceous and woody shoots), a phenomenon that may be exacerbated by the use of Gelrite™ at low concentrations (Kevers *et al.*, 2004). Whilst not the most inspiring of topics, the use of

setting agent has been reported to affect plant tissue culture. Garin *et al.* (2000) noted an increase in successful generation of somatic embryos of *Pinus strobus* on 1% Gelrite™ media compared with 0.6% media. Additionally, Owens & Wozniak (1991) were able to minimise significant variation in shoot and somatic embryo generation from sugarbeet callus by optimising the concentration of selected agent (e.g. 0.12% Gelrite™ but 0.7% Bacto agar) to obtain an equal gel matrix potential. Similarly, shoot regeneration of *Aloe polyphylla* was significantly hampered by the use of Gelrite™ compared to agar-based media (George *et al.*, 2008a; Ivanova & Van Staden, 2011). The use of Gelrite™ at a sub-optimal concentration could explain the moist or hyperhydric FEC produced during this investigation. Noble agar, extracted from species of red algae and the purist of the Difco™ brand agars, instead allowed consistent production of desirable FEC, as well as improving somatic embryo generation. Ergo, all plated media was prepared with Noble agar with the exception of CBM where no discernible morphological difference was observed in plantlets grown in Gelrite™ containing media. Other setting agents used in cassava transformation include phytigel (Sigma-Aldrich; Schöpke *et al.*, 1996) and microagar (Duchefa; Schreuder *et al.*, 2001) but these were not investigated due to the excellent results achieved with Noble agar.

The advanced climate regulation system in the Sanyo MLR Plant Growth Incubator was superior for all stages of tissue culture, compared to the Climate Controlled Room and General Plant Growth Chamber (Weiss Kallenkamp). Most noticeable was the large amount of moisture collecting on the lids of the culture dishes, which inevitably led to the production of mushy FEC. To what extent this affected FEC growth was not determined but it was hypothesised that there would be changes to media conditions (e.g. pH, nutrient concentrations, etc), as well as light penetration. GUS assays using FEC transformed with pCAMBIA 1305.1 also revealed a slight improvement in transformation capacity of tissue maintained in the Sanyo MLR Plant Growth Incubator. The production and long-term maintenance of high quality FEC is paramount to reduce somaclonal variation; Raemakers *et al.* (2001) demonstrated FEC maintained for more than two years generated into plants with reduced vigour and greyish leaf colour compared to those from FEC that had been cultured for up to six months. Crucially, the adoption of Noble agar and advanced climate chambers ensured persons who had limited experience of tissue culture were able to generate FEC of mediocre quality that could still be transformed and regenerated into healthy plants.

4.4.2 Culturing in SH liquid media altered FEC morphology and increased the likelihood of microbial contamination

The problems associated with cultivating material in liquid media, namely morphological changes to FEC and microbial contamination, posed the most serious bottleneck in cassava tissue culture. Although liquid media has been used successfully to generate transgenic cassava (Raemakers *et al.*, 2005; Zhang *et al.*, 2005; Zhang *et al.*, 2010), in this study contamination caused losses of 50-100% and FEC consistently failed to regenerate on hygromycin-containing MSN. Interestingly, liquid media stages have been abandoned by N. Taylor (*pers. comm.*) who first developed FEC *via* this method (Taylor *et al.*, 1996). Given the problems experienced, the transformed *Agrobacterium* was pipetted directly onto the FEC propagation plates, a technique also used in the transformation of *Brachypodium distachyon* (a temperate grass; Alves *et al.*, 2009). The supposed role of SH media is to induce maturity of the FEC (P. Zhang, *pers. comm.*), which correlates with the observed changes in morphology. The morphological changes to FEC are possibly explained by auxin content, physical damage *via* the sieving process and also due to the characteristics of liquid cultures. The altering of gene expression to maintain an embryogenic programme has been linked to DNA methylation and influenced by auxin (e.g. picloram). A degradation or sub-optimal concentration of auxin could have influenced the transition from FEC to early embryogenesis observed with the formation of globular units. Stress has also been proposed to mediate signal transduction cascade leading to changes to gene expression (George *et al.*, 2008b). Additionally, an imbalance in water potential of the media may result in distended, hyperhydric FEC and also oxygen content in the solution may be insufficient for the submerged tissue (George *et al.*, 2008a). The duration of FEC in SH liquid media may have been too long since torpedo structures and sometimes even cotyledons were observed. Indeed, the cultivation of secondary somatic embryos in SH liquid media, supplemented with 12 mg L⁻¹ picloram, prior to transfer to auxin-free media for germination, has been reported to be the most efficient method for embryo development (Groll *et al.*, 2001). Whilst all these factors raise questions regarding cassava somatic embryogenesis, optimisation of the liquid media stages was disregarded due to the propensity for contamination in the sucrose rich media and was unnecessary given the accomplishments of culturing FEC on GD plates only.

Acclimatising FEC on GD plates supplemented with carbenicillin and low concentrations of hygromycin following co-cultivation markedly improved regeneration capacity. Similar

strategies were described by Zhang & Gruissem (2004) but using SH liquid media and Schreuder *et al.* (2001) who used a combination of liquid media and plates for post co-cultivation maturation. The acclimatisation process on GD used in this study clearly enabled the FEC to effectively express genes in the T-DNA (e.g. *hptII*) whilst on selection media that previously was too stringent. Raemakers *et al.* (2001) also demonstrated the significance of maturation using the *luc* reporter gene following biolistic bombardment of FEC. Extended acclimatisation *via* the weekly transfer of co-cultivated FEC onto media supplemented with increasing amounts of antibiotic (GD+H5 → GD+H8 → GD+H15) also assisted FEC recovery and encouraged development on MSN+H20.

Suspected transformed FEC were originally transferred by hand from GD to MSN but this procedure proved hugely laborious. Alternative strategies such as spreading material on sterile filter paper and nylon membrane were tested, but most effective was the use of sterilised nylon mesh. The co-cultivated FEC could be spread and efficiently transferred to the necessary media on a weekly basis without the membrane disintegrating (data not shown). This also prevented disruption to material, minimised fluctuation in nutrient and antibiotic concentrations and also reduced the risk of contamination. It transpired that spreading FEC thinly was crucial to prevent accumulation of NEFC, especially on the GD-based media, and also to lessen *Agrobacterium* growth. The cotyledon structures that developed following several cycles on MSN were transferred to media to promote shoot formation (CEM). *Agrobacterium* growth around the developing cotyledons on CEM could seriously hamper growth of the shoot but this was easily suppressed with the inclusion of 100 mg L⁻¹ carbenicillin (CEM+C100).

4.4.3 Optimised antibiotic concentration is crucial for efficient FEC selection and regeneration

The results from the rooting experiment (Section 4.3.4) suggests that a maximum concentration of approximately 10 mg L⁻¹ hygromycin is sufficient to screen *in vitro* cuttings. This is 4-fold less than the advised concentration (40 mg L⁻¹; P. Zhang *pers. comm.*), although in keeping with published data that recommended 8 mg L⁻¹ (Zhang *et al.*, 2000a; Zhang & Puonti-Kaerlas, 2004). Similarly, an appropriate concentration of antibiotic for selective regeneration in MSN plates was 10-15 mg L⁻¹, which is comparable with data from Schreuder *et al.* (2001) who studied the increase in weight of non-transformed FEC plated on media supplemented with various concentrations of hygromycin, paramomycin and kanamycin. Curiously, Schöpke *et al.* (1996) recorded

FEC survival on media supplemented with as much as 175 mg L⁻¹, although the relatively short test duration (one week) may have affected interpretation; according to Zhang *et al.*, (2000b) 96% of non-transformed suspension cells were killed at 50 mg L⁻¹ within four weeks. Data presented in this thesis identified that over a period of approximately five weeks even non-transformed FEC can regenerate whilst on media supplemented with 15 mg L⁻¹ hygromycin. This suggests that changes in media conditions do occur (possibly a degradation of the antibiotic) and hence why media was replenished on a weekly basis.

With the remarkable improvement in regeneration of transformed FEC as gauged by transformation with pCAMBIA 1305.1 and GUS assays, it became apparent that media for embryo development (CMM) and shoot regeneration (COM), outlined in Zhang & Gruissem (2004) and Section 2.5.2, were redundant. Instead, transfer of established green cotyledons directly to CEM+C100 and then cycled on fresh media every two weeks induced large numbers of shoots and plantlets with a normal phenotype (please see following chapters for further details). Omission of the CMM and COM stages not only simplified the protocol but also ensured the developing material was not subject to repeatedly changing environments. The numerous adaptations radically improved efficiency of the transformation protocol (Figures 4.14 and 4.15; Bull *et al.*, 2009) and are briefly summarised below:

- Omission of SH liquid media stages both prior to and following co-cultivation of FEC and *Agrobacterium*.
- Acclimatisation of co-cultivated FEC on GD media with increasing concentration of hygromycin.
- Optimisation of hygromycin concentration in regeneration media (MSN) and for rooting assay.
- Extensive use of nylon mesh to support co-cultivated FEC on regeneration media and allowing frequent transfer of material to freshly prepared media.
- Use of Noble agar in all media (except CBM) to prevent hyperhydric tissue.
- Use of an advanced climate chamber (Sanyo MLR Plant Growth Incubator).
- Direct inoculation of FEC on GD propagation media.
- Omission of CMM and COM stages, replaced with cycling of material on CEM to establish juvenile shoots.

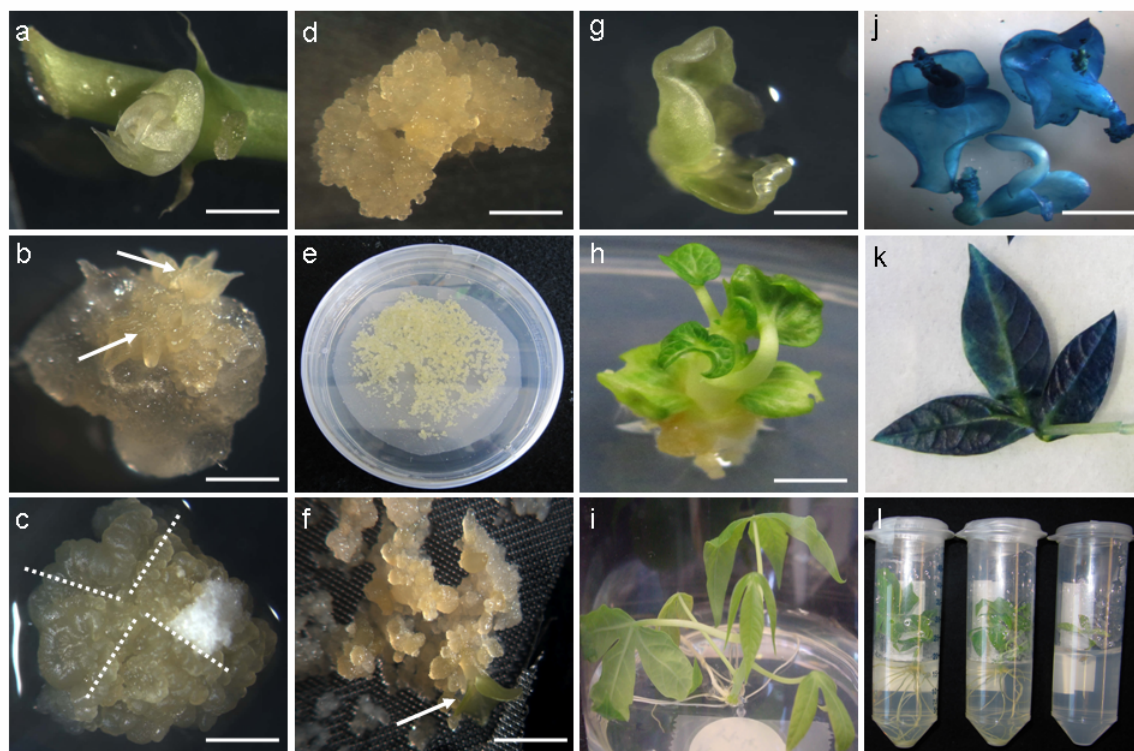


Figure 4.14 Procedure for generating transgenic cassava plants. Swollen axillary bud on CAM **(a)**. Primary somatic embryos (indicated by arrows) developing on a bed of NEFC on CIM **(b)**. Maturing somatic embryos on CIM. Dashed line indicates approximate suggested division for further propagation **(c)**. Cluster of FEC on GD appropriate for *Agrobacterium* inoculation **(d)**. FEC following co-cultivation spread onto mesh on GD+C250 **(e)**. Developing embryo/cotyledon (indicated by arrow) on MSN+C250+H15. Transformed FEC seen as swollen, yellowish structures. Non-transformed are smaller, white clusters **(f)**. Developing embryo/cotyledon transferred to CEM+C100 **(g)**. Appearance of immature shoots following several weeks on CEM+C100 **(h)**. *in vitro* transgenic cassava plantlet **(i)**. Developing embryos/cotyledons from MSN+C250+H15 used for GUS assay. Blue precipitate clearly visible throughout all tissue **(j)**. GUS assay of leaves **(k)**. Rooting assay of transgenic plantlets (left and centre) and wild-type TMS60444 (right) on CBM+C50+H10 **(l)**. Scale bar is 5 mm. Figure from Bull *et al.* (2009).

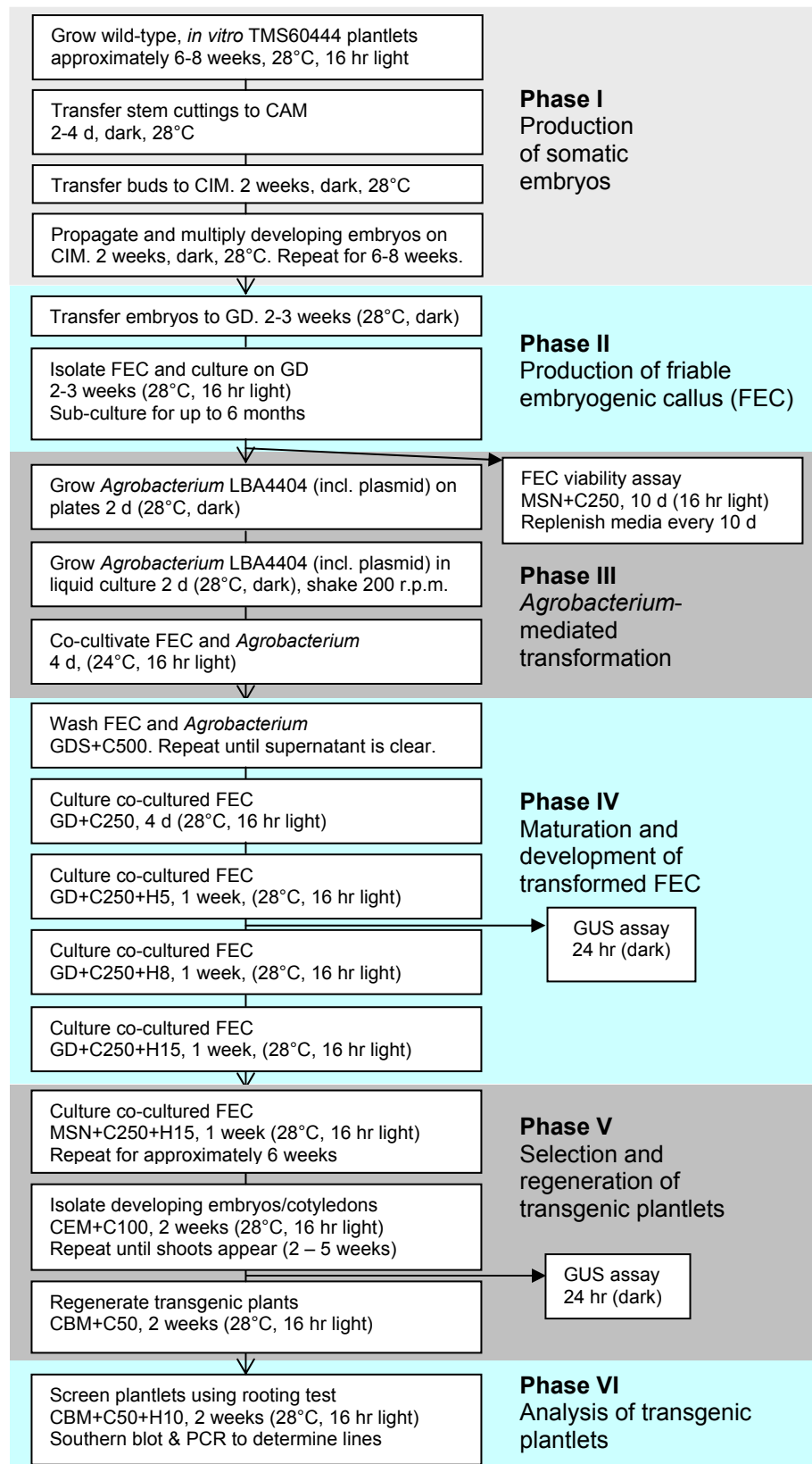


Figure 4.15 Overview of *Agrobacterium*-mediated transformation of FEC from cassava cultivar TMS60444. For detailed information please refer to Bull *et al.* (2009).

5 CASSAVA TRANSFORMATION WITH ASCORBATE PEROXIDASE

5.1 INTRODUCTION

Ascorbate peroxidase (APX; EC. 1.11.1.11) is an enzyme found mainly in photosynthetic plants and algae. It uses ascorbate (ascorbic acid or vitamin C) as a reducing agent to convert H_2O_2 to water and monodehydroascorbate, forming part of the ascorbate-glutathione pathway (Asada, 1999). It has a high affinity to H_2O_2 , more so than CAT where effective removal of H_2O_2 relies upon a high concentration of enzyme at specific locations (Mittler & Poulos, 2005). APX is encoded by a small gene family, which is more closely related to yeast and bacterial (class I) peroxidases than classical plant (class III) peroxidases (Mittler & Zilinskas, 1991; Passardi *et al.*, 2007), with seven genes identified in tomato (Najami *et al.*, 2008), eight in rice (Teixeira *et al.*, 2006) and nine in *Arabidopsis* (Mittler *et al.*, 2004). Found in various cellular compartments, APX in *Arabidopsis* has been described in the thylakoid membrane (tAPX) and stroma (sAPX) of the chloroplast, microsomal bodies (mAPX) i.e. in peroxisomes and glyoxysomes, the cytosol (cAPX) and also in mitochondria (mitAPX; Mittler *et al.*, 2004). Recently, Panchuk *et al.* (2005) revealed that APX expression is differentially regulated during leaf senescence in *Arabidopsis*, suggesting functional specialisation of the different isoenzymes. To date, whilst it is presumed cassava also contains different types of APX, only a cytosolic form (*MecAPX2*; NCBI accession AY973622) has been identified (Gómez-Vásquez *et al.*, 2004).

Although expression and regulation of APX in cassava has not been fully elucidated, a microarray analysis using cultivar CM2177-2 revealed that expression of *MecAPX2* was up-regulated following root harvest. Expression levels peaked (1.7-fold increase) at 24 hr post-harvest before returning to basal levels (Reilly *et al.*, 2007). Isamah (2004) also showed that POX activity in cassava roots (cultivar Oyolu) peaked at 24 hr post-harvest, although data was not provided as to the precise expression of APX. A *MecAPX2* cDNA probe corresponding to the EST identified in the microarray analysis was used in Northern blot hybridisation to corroborate these findings (Reilly *et al.*, 2007). However, this revealed peak expression occurred later after harvest (between 48 hr and 72 hr), a discrepancy that was also observed with other genes tested e.g. *MecCAT2* (NCBI accession AY973614). This was suggested to be an artefact of the experimental design

since the microarray analysis was performed in Colombia using field-grown roots, where the onset of PPD symptoms was more rapid than in the UK glasshouse-cultivated roots that were used for Northern blot analysis. Despite the discrepancy, it was concluded that *MecAPX2* expression is up-regulated during PPD and thus serves an important role in regulating H₂O₂ accumulation.

Despite scant information regarding APX in cassava, studies involving over-expression and antisense/gene silencing constructs in other plant species proffer an insight into the role of APX in the ROS-scavenging pathway. Over-expression of the *ASCORBATE PEROXIDASE-LIKE 1* gene from *Capsicum annuum* (pepper) in transgenic tobacco resulted in increased plant growth and also improved tolerance to methyl viologen- (MV, a superoxide generating compound) mediated oxidative stress (Sarowar *et al.*, 2005). Unlike the aforementioned study, many projects that seek to modify plant defence rely on multigene constructs, utilising several genes in the ROS-defence pathway. For example, over-expression of *APX* and *SOD* in chloroplasts and driven by the oxidative stress inducible *SWEET POTATO PEROXIDASE ANIONIC 2* (*SWPA2*) promoter, led to enhanced tolerance to MV-mediated stress in potato plants (Tang *et al.*, 2006). The same construct used to transform tall fescue plants also resulted in decreased ROS accumulation in response to abiotic stress (Lee *et al.*, 2007). Conversely, a wheat *tAPX* knockout mutant led to a 40% decrease in activity and experienced reduced growth and photosynthetic activity (Danna *et al.*, 2003). An antisense of *tAPX* in *Arabidopsis* also led to a 50% reduction in enzyme activity and increased sensitivity to the superoxide-inducing herbicide paraquat (Tarantino *et al.*, 2005), whilst an antisense *cAPX* resulted in tobacco plants with reduced tolerance to high light and oxidative stress (Örvar & Ellis, 1997). These selected studies and basic knowledge of APX in cassava clearly show the important role of the enzyme and raises the possibility that over-expression of *APX* may improve modulation of H₂O₂ in transgenic cassava.

5.2 RESEARCH OBJECTIVES

The goal is to transform FEC of TMS60444 with the over-expression construct pDEST™-*MecAPX2* and the antisense construct pDEST™-antiAPX to generate transgenic plants. The *StPAT* promoter used to regulate transgene expression in the constructs should result in expression predominantly in root tissue. Transgene expression profiles and enzyme activity will be measured in independent transgenic lines and the harvested storage roots will be assessed for delayed PPD.

5.3 RESULTS

5.3.1 Putative APX genes in cassava

Nucleotide BLAST searches (Section 2.4.10) using the nine *APX* sequences from *Arabidopsis* led to the identification of seven orthologous transcripts in cassava, which clustered in a phylogenetic tree according to predicted cellular location (Figure 5.1). *MecAPX1* and *MecAPX2* cluster with *Arabidopsis* genes that are reportedly located in the cytosol, whereas *MecAPX3* groups with genes expressed in microsomal bodies. Only one transcript of a chloroplastic *APX* could be identified in cassava (named here as *Mec-chlAPX*). The functional characteristics of *AtAPX4* and *AtAPX6* (and thus the cassava orthologs) are unclear but are possibly located in the cytosol. BLAST search for orthologs of *AtAPX3* and *AtAPX5* derived the same transcript in cassava. Similarly transcripts of *MecAPX1* and *MecAPX7* were identical except for a 3 bp insertion (valine) in the latter (Figure 5.1). Collectively, these data indicate that *APX* exists as a small gene family in cassava and transcripts have been putatively identified that are likely to function in several cellular locations.

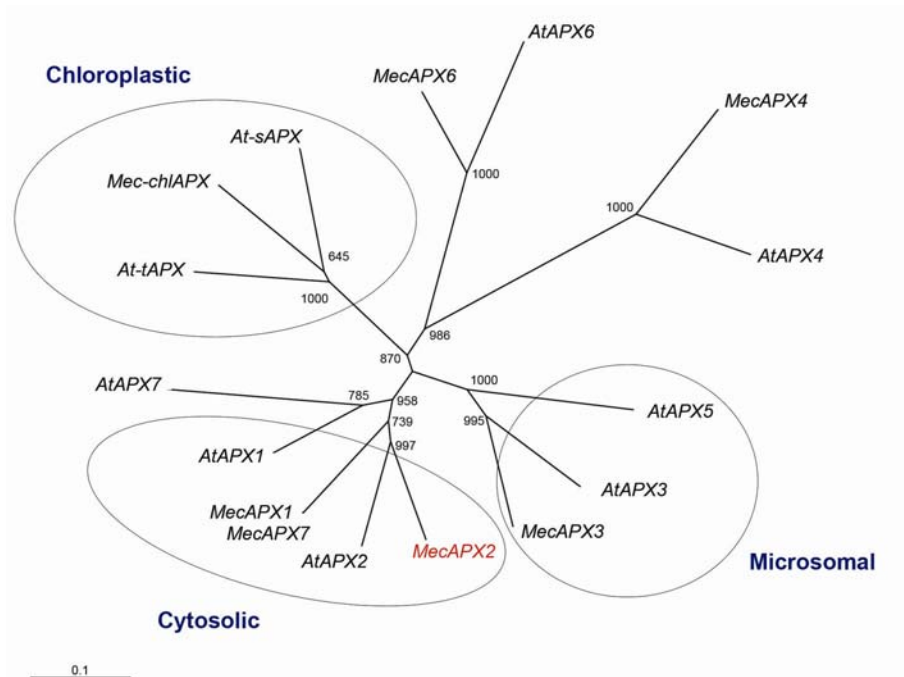


Figure 5.1 Phylogenetic tree of *APX* nucleotide sequence from *Arabidopsis* and cassava.

Arabidopsis sequence obtained from TAIR database: *AtAPX1* (AT1G07890), *AtAPX2* (AT3G09640), *AtAPX3* (AT4G35000), *AtAPX4* (AT4G09010), *AtAPX5* (AT4G35970), *AtAPX6* (AT4G32320), *AtAPX7* (AT1G33660), *At-tAPX* (AT1G77490) and *At-sAPX* (AT4G08390).

Cassava sequence (sourced from Phytozome database): *MecAPX1* & *MecAPX7* (cassava4.1_014642m & cassava4.1_014423m), *MecAPX2* (cassava4.1_024509m), *MecAPX3* & *MecAPX5* (cassava4.1_013189), *MecAPX4* (cassava4.1_027646m), *MecAPX6* (cassava4.1_018316), *Mec-chlAPX* (cassava4.1_009867m). Groups based on predicted cellular locations (circled) and gene selected as transgene (red font). Bootstrap values (1000 replicates) shown.

5.3.2 Successful generation of pDEST™-*MecAPX2* transgenic cassava

Independent batches of FEC (FEC6, FEC9 and FEC10, cultivar TMS60444) were transformed as described by Bull *et al.* (2009) using pDEST™-*MecAPX2* to successfully generate approximately 150 *in vitro* plantlets. The plantlets were catalogued into four groups (Group A → D) depending on the batch of FEC used and date transformed and then assigned a letter (A → Z and subsequently AA → AZ if more than 26 plants from a particular group were produced). For example, APX:CF refers to the sixth plant labelled from Group C, whilst APX:DAF is the 32nd plant labelled from Group D. Successful expression of *hptII* integrated into the plant genomes was confirmed *via* a rooting assay (Bull *et al.*, 2009), with only five samples (3% of tested plantlets) failing to establish roots and thus presumed non-transgenic and discarded. 30 plantlets representing each of the four groups were selected and maintained for further analyses. Genomic DNA was extracted from *in vitro* leaf tissue (Section 2.3.1) and used in PCR-amplifications using primers to *hptII* (Hygro-For & Hygro-Rev; Table 2.1) and *StPAT* promoter/transgene (DESTSeqF1 & apxR1, Table 2.1; Section 2.1.2). The target *hptII* sequence was successfully amplified in all samples, with the exception of APX:AA, APX:AN and APX:DAE, yielding a product of approximately 1 Kb (Figure 5.2). Similarly, the predicted sized product (also approximately 1 Kb) using DESTSeqF1 & apxR1 primers was obtained from almost all samples with the exception of APX:AA and APX:CAD (Figure 5.2). It is noteworthy that 28 plantlets transformed with the respective antisense construct (pDEST™-antiAPX) also successfully passed the rooting test but due to time restrictions they were retained as *in vitro* stocks and not analysed further.

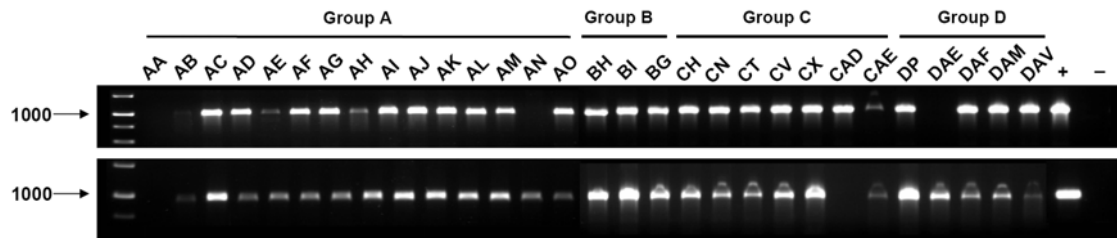


Figure 5.2 PCR-amplification products using pDEST™-MecAPX2 transformed *in vitro* plantlets. Products using *hptII* specific primers (Hygro-For & Hygro-Rev) (**upper image**) and StPAT promoter/transgene primers (DESTSeqF1 & apxR1) (**lower image**). Products from plasmid DNA (+) and genomic DNA from wild-type TMS60444 (–) are shown. DNA ladder (bp) indicated. Plants grouped according to batch of FEC used and date transformed (Group A → D).

Genomic DNA isolated from the transgenic and wild-type *in vitro* plantlets was digested using *HindIII* and electrophoresed (Section 2.4.11) for Southern blot hybridisation with a DIG-labelled *hptII*-annealing probe (Sections 2.4.13 and 2.4.14). All samples, with the exception of APX:CAE, hybridised to the probe (Figure 5.3) indicating stable integration of the T-DNA in the plant genome. Interestingly, only one or two genomic fragments were hybridised in 77% and 23% of samples, respectively. These data suggest minimal T-DNA integration, although precise transgene copy number was not determined. Approximately eight independent lines could be identified amongst plants in Group A alone (Figure 5.3a) and 10 lines were identified from Groups B, C and D collectively (Figure 5.3b). It is conceivable that some of the lines in Group A are the same as those identified in Groups B, C and D. For this to be irrevocably confirmed it would be necessary to hybridise all predicted independent lines on a single membrane. However, due to time constraints further analysis was based solely on the plant lines from Groups B, C and D.

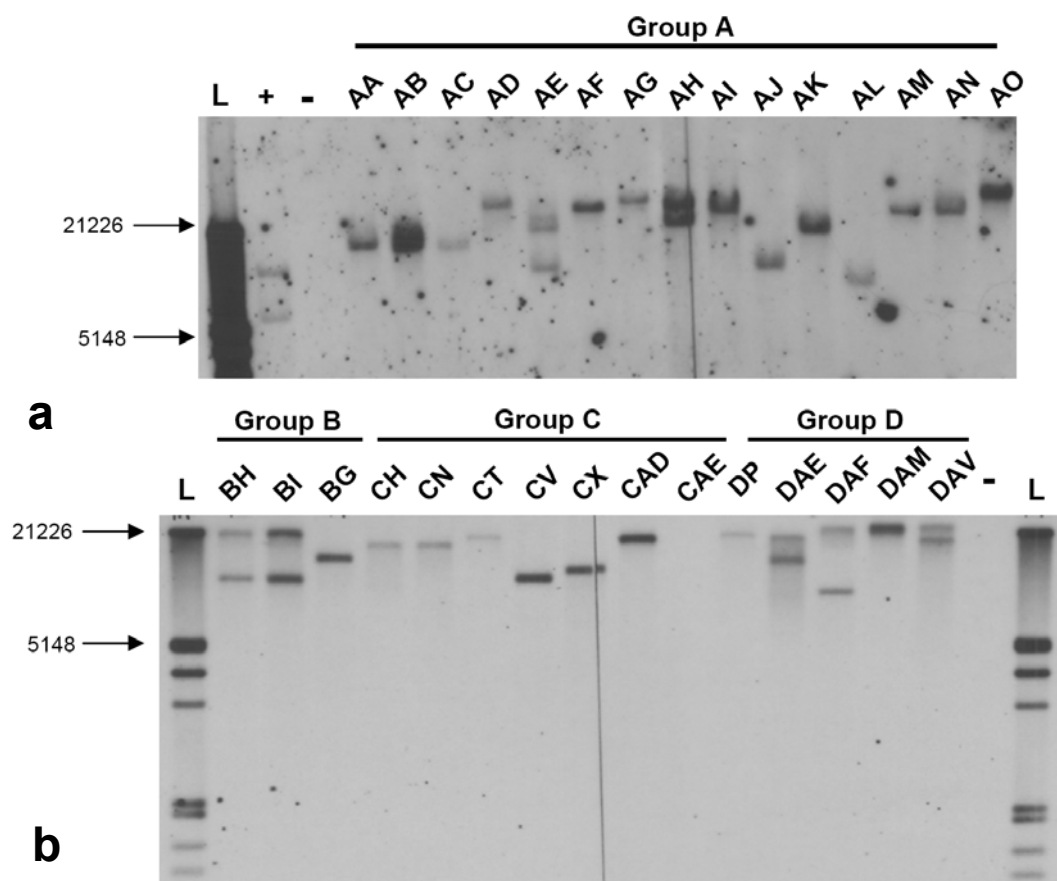


Figure 5.3 Southern blot hybridisation of *Hind*III digested genomic DNA from *in vitro* plantlets transformed with pDEST[™]-*MecAPX2*. Transformation Group A (a) and transformation Groups B, C and D (b). Samples hybridised to a *hptII*-annealing DIG-labelled probe. DNA ladder (bp; lanes L), plasmid DNA (+) and wild-type TMS60444 genomic DNA (–) shown.

Plantlets from the 10 independent lines identified in Group B (APX:BG and APX:BH), Group C (APX:CH, APX:CT, APX:CV and APX:CX) and Group D (APX:DAE, APX:DAF, APX:DAM and APX:DAV) were multiplied *in vitro* and approximately five plantlets per line were then transferred to soil to establish plants in the glasshouse (Section 2.5.3).

5.3.3 Morphology of pDEST[™]-*MecAPX2* transgenic plants

25 pDEST[™]-*MecAPX2* transgenic and three wild-type TMS60444 plants were successfully established in the glasshouse (Table 5.1) and produced true storage roots (albeit smaller than field-grown roots). This is the first occasion large scale propagation of pot-based storage roots has been achieved at the University of Bath. Furthermore, all

plants had a normal phenotype (Figure 5.4), lacking abnormalities often associated with poor plant husbandry or genetic mutations (somaclonal variation) in cassava e.g. thickened stems, irregular leaf development, yellow/grey leaf colour and stunted growth. Unfortunately some material was lost due to microbial contamination of media during propagation of *in vitro* plantlets, as well as failings with the systems that regulate environment conditions. This resulted in a varying number of plants that were available for analysis per line (Table 5.1). Lines APX:DAE and APX:DAM incurred the greatest loss and are represented by two plants each. Additionally, due to a sampling error, data collected for lines APX:BH and APX:CH were disregarded.

Plant height was measured from the root stock to apical growth tip and in some cases was variable between plants of the same line. Most dramatically APX:DAE plant one was 37 cm in height whilst plant two was 92 cm. However, overall the mean plant height of independent lines were commensurate with wild-type data (Figure 5.5) as determined by independent-samples *t*-test (Table 5.1).

Table 5.1 Morphology of glasshouse cultivated plants.

Plant line	Number of plants	Mean plant height (cm), S.E. & (max. / min.)	<i>t</i> -test [†]	Number of roots	Mean root stock weight (g), S.E. & (max. / min.)	<i>t</i> -test [†]
APX:BG	4	69.5, 6.96 (86 / 52)	p=0.759	8	55.8, 3.35 (65 / 49)	p=0.187
APX:CT	3	88.3, 3.18 (92 / 82)	p=0.341	5	56.7, 6.01 (65 / 45)	p=0.286
APX:CV	3	70.0, 8.08 (84 / 56)	p=0.811	5	51.0, 2.08 (55 / 48)	p=0.515
APX:CX	4	73.3, 6.97 (86 / 56)	p=0.975	8	64.3, 6.09 (82 / 55)	p=0.093
APX:DAE	2	64.5, 27.50 (92 / 37)	p=0.744	3	64.0, 6.00 (70 / 58)	p=0.102
APX:DAF	3	85.7, 0.68 (87 / 85)	p=0.417	5	57.0, 12.01 (81 / 44)	p=0.504
APX:DAM	2	76.0, 2.00 (78 / 74)	p=0.889	4	53.5, 11.50 (65 / 42)	p=0.703
APX:DAV	4	77.5, 5.62 (89 / 63)	p=0.761	6	52.5, 5.25 (67 / 43)	p=0.528
TMS60444	3	73.7, 11.84 (86 / 50)	n/a	5	47.7, 4.18 (56 / 43)	n/a

[†] independent-samples *t*-test (wild-type *versus* transgenic line), significance $p \leq 0.05$ (*)

n/a not applicable (wild-type cassava, cultivar TMS60444)



Figure 5.4 pDEST™-*MecAPX2* transgenic plant. Whole plant (a) and root stock (b). Plant photographed five months after transfer of *in vitro* plantlet to soil and maintenance in glasshouse. Examples typical of growth for both transgenic and wild-type material.

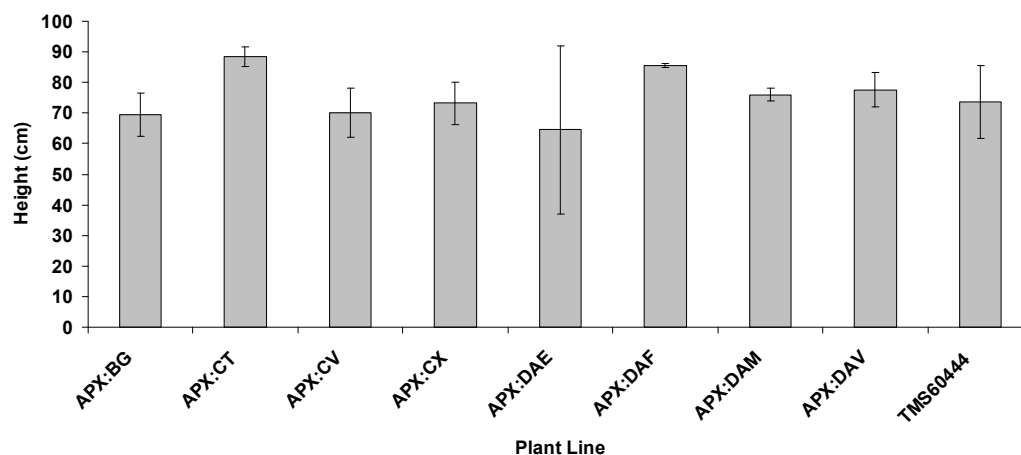


Figure 5.5 Mean height of glasshouse cultivated plants. Eight independent lines of pDESTTM-*MecAPX2* transgenic plants and wild-type cassava (TMS60444) were assessed. S.E. shown.

An average of 1.75 roots were harvested from each plant with a mean root stock weight of 55.8 g (including wild-type data). As for plant height, there was no statistically significant difference between the mean weight of root stocks from transgenic plants compared with wild-type material, as determined by independent-samples *t*-tests (Table 5.1; Figure 5.6). Plants invariably produced storage roots of varying size with one larger root and then two or three thinner roots, as can be seen in Figure 5.4b.

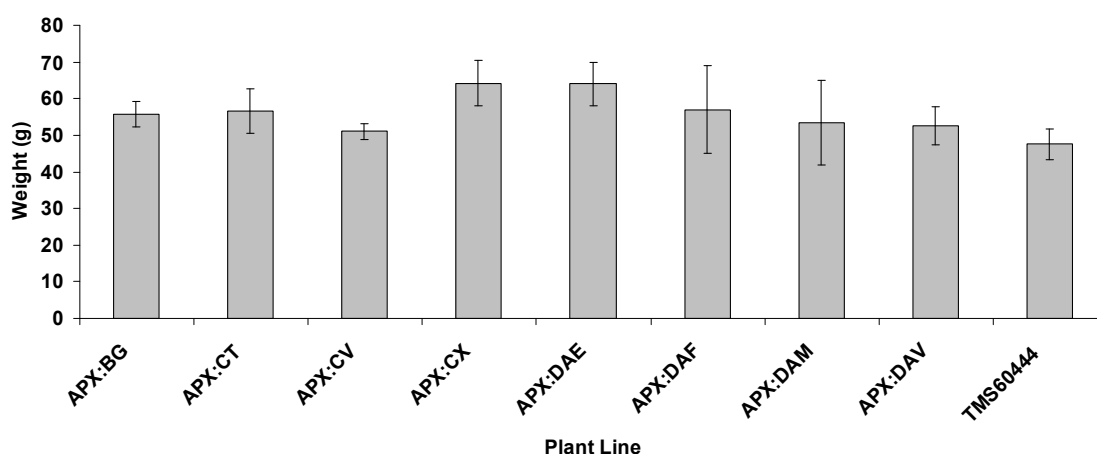


Figure 5.6 Mean root stock weight of glasshouse cultivated plants. Eight independent lines of pDESTTM-*MecAPX2* transformed plants and wild-type cassava (TMS60444). S.E. shown.

5.3.4 Trial experiments to devise a PPD assay for glasshouse cultivated roots

The visual assessment of PPD symptoms in field-grown storage roots has been described and tend to follow two different approaches. The first, denoted here as the “whole root method”, requires trimming the proximal and distal ends that are then covered with cling film before storing the roots away from direct sunlight at ambient conditions. In tropical and sub-tropical countries where this method has been used this is usually 28°C and >80% humidity. At designated time points, randomly selected roots are sliced, the symptoms of each slice scored and a mean score for the root calculated (van Oirschot *et al.*, 2000; Wheatley *et al.*, 1985). The second approach (referred to here as the “harvest and slice method”) necessitates slicing the root(s) immediately after harvest, incubating the material (as above) and then scoring symptoms of different slices at selected time points, which are usually between 0 hr and 4-5 d. Overall, the former technique is considered more representative of harvesting and thus PPD but relies upon a plentiful supply of roots for analysis. Unfortunately there are no reports of suitable procedures to assess PPD in the smaller storage roots of glasshouse-cultivated plants. Therefore trial experiments adopting these two approaches were undertaken for this investigation using a further 21 roots of the 10 independent lines of pDEST™-MecAPX2 transformed plants (Table 5.2).

Table 5.2 pDEST™-MecAPX2 transgenic glasshouse-cultivated roots used for assessing techniques to score PPD symptoms.

Transformation group	Plant line	Number of harvested roots
Group B	APX:BG	1
	APX:BH	4
Group C	APX:CH	2
	APX:CT	1
	APX:CV	3
	APX:CX	2
	APX:DAE	2
Group D	APX:DAF	1
	APX:DAM	4
	APX:DAV	1

5.3.4.1 “Harvest and slice” method

A selection of glasshouse-cultivated roots were harvested (Table 5.2), sliced approximately 5 mm in thickness and placed on moist filter paper in Petri dishes to minimise desiccation. Material was incubated in a plastic box at 26°C and 1 ml of SDW was used to remoisten the filter paper every 48 hr. Material was sampled and photographed at 0, 6, 12, 24, 48, 72 and 96 hr post harvest. No or only minimal PPD symptoms were observed in tissue at 0 hr and 6 hr post-harvest (Figures 5.7a and 5.7b) but deterioration (light brown discolouration) was more apparent in tissue assessed 12 hr post-harvest (Figure 5.7c). By 24 hr post-harvest the root slices were clearly deteriorated with discolouration seen mainly in the cortical parenchyma (Figure 5.7d). The storage parenchyma also developed brown discoloration but usually became white/grey and desiccated - symptoms that were repeated in tissue at 48 hr, 72 hr and 96 hr post-harvest (Figure 5.7e-g). Interestingly, these symptoms appeared akin to general deterioration and desiccation rather than the expected blue/black vascular streaking synonymous with PPD observed in field-grown roots.

Further small-scale trials were completed to understand better whether the placing of root slices directly on moistened paper was affecting symptom development. For example, slices were placed on dry filter paper and a pot of water in the storage box provided humidity. In theory, this approach should provide a more constant environment, minimising fluctuations in humidity, but unfortunately material developed symptoms similar to those seen in Figure 5.7a-g and thus was not considered a significant improvement to the technique. A further modification tested root samples incubated at ambient room conditions but they became highly desiccated within 6-12 hr (data not shown). In summary, whilst the “harvest and slice” method may be used for field-grown storage roots, the thinner roots from glasshouse-cultivated plants are seemingly too prone to desiccation. Additionally, it was highly laborious and difficult to sample tissue at the selected time points.

5.3.4.2 “Whole root” method

The limited availability of glasshouse-cultivated roots meant this technique required modification from the outset. Rather than using intact roots, as has been documented with field-grown material (van Oirschot *et al.*, 2000), the roots were divided into two and placed on Petri dishes in a plastic box for incubation at 26°C. A slice of root was also removed to represent 0 hr collected material. One half of the root was removed,

desiccated tissue was trimmed from the end and symptoms observed at 24 hr post-harvest, whilst the second half was incubated and assessed 96 hr post-harvest. In general, mild or no PPD symptoms were observed in tissue sampled 24 hr post-harvest but symptoms were clearly evident 96 hr post-harvest (Figure 5.7h). Significantly, incubating partially intact roots ensured the tissue was not desiccated and symptoms were more typical of PPD i.e. vascular streaking. The findings from the trial experiments discussed here led to the implementation of the technique described in Section 2.5.4 and which was applied for the assessment of PPD presented in this chapter.

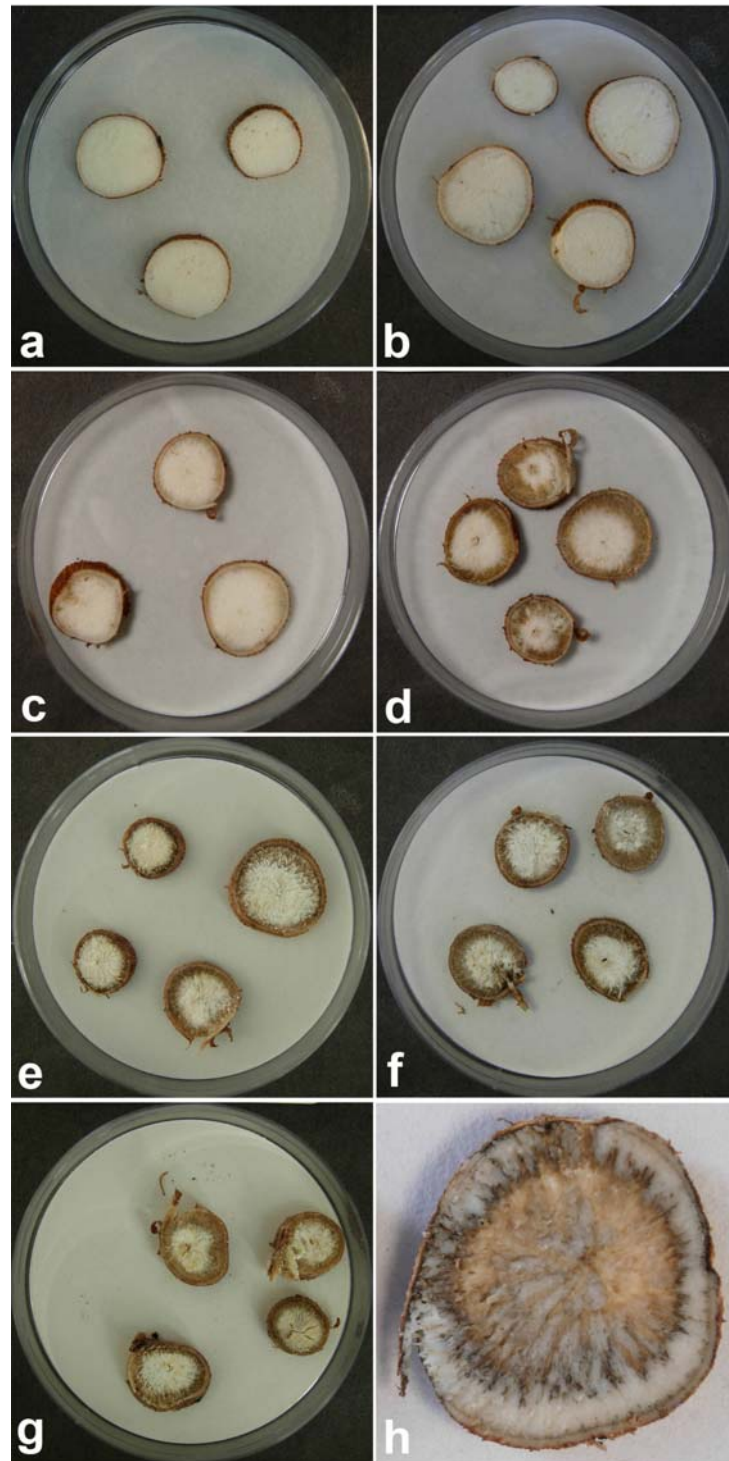


Figure 5.7 Roots assessed for PPD symptoms. APX:TAD root slices at 0 hr (a), 6 hr (b), 12 hr (c), 24 hr (d), 48 hr (e), 72 hr (f) and 96 hr post-harvest (g). Roots incubated on moist filter paper in a covered box at 26°C, dark. 90 mm diameter Petri dishes used in experiments (a-g). Cross-section of a halved root at 96 hr post-harvest and incubated at 26°C, dark. Note streaking symptoms rather than general deterioration observed in other samples (h). Roots harvested from glasshouse cultivated plants.

5.3.5 PPD assay of pDEST™-MecAPX2 transformed plant lines

44 glasshouse-cultivated storage roots from transgenic plants and five roots of wild-type TMS60444 were harvested (Table 5.1) and symptoms of PPD assessed at 0 hr, 24 hr and 96 hr post-harvest. Root nomenclature is as follows – expression construct : plant line : plant & root number. For example, line APX:DAE had two plants, plant A yielded only one suitable root (APX:DAE:AR1), whereas plant B produced two roots (APX:DAE:BR1 and APX:DAE:BR2; Figure 5.8). In all samples (both transgenic and wild-type) no PPD symptoms were observed at 0 hr but discolouration was visible in some roots at 24 hr (Figures 5.8, 5.9 and 5.10). However, symptoms were not necessarily consistent between roots from within lines or even from the same plant. For example, root TMS60444:AR1 has no symptoms whilst TMS60444:AR2 displayed mild symptoms of PPD at 24 hr post-harvest (Figure 5.9). Similar results were observed with transgenic material - APX:DAE:BR2 has mild PPD symptoms at 24 hr post-harvest but a root from the same plant (APX:DAE:BR1) was symptomless (Figure 5.8). At 96 hr post-harvest, all transgenic (Figures 5.8 and 5.10) and wild-type roots (Figure 5.9) had developed severe PPD symptoms. It is important to note that although some roots (e.g. APX:DAE:BR2; Figure 5.8) appeared resistant to PPD at 96 hr post-harvest, the material was in-fact highly desiccated and chalky in texture. This possibly reflects general deterioration rather than PPD.

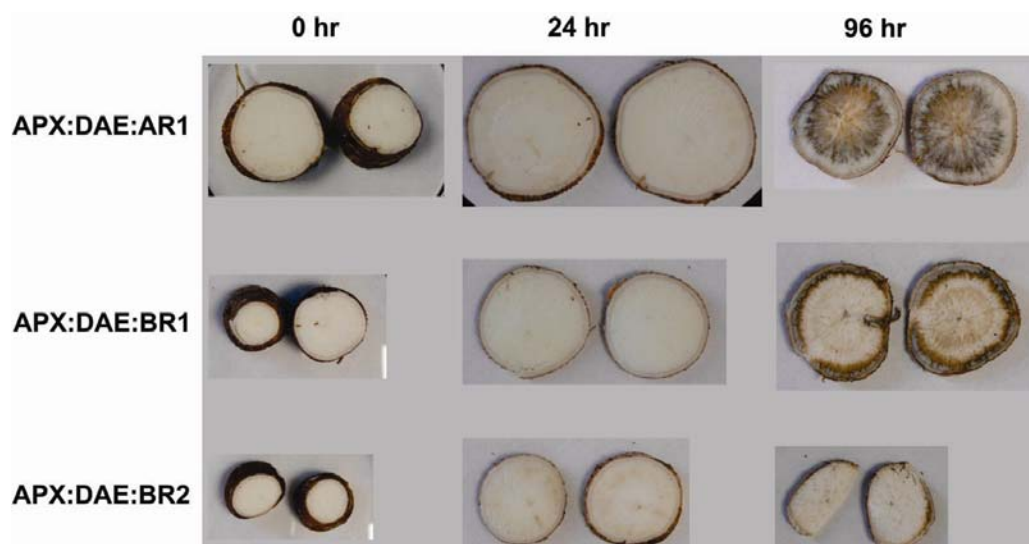


Figure 5.8 Transgenic APX:DAE roots following harvest. Sections of roots shown 0 hr, 24 hr and 96 hr post-harvest. Three roots were harvested and assessed from two plants (APX:DAE:A and APX:DAE:B).

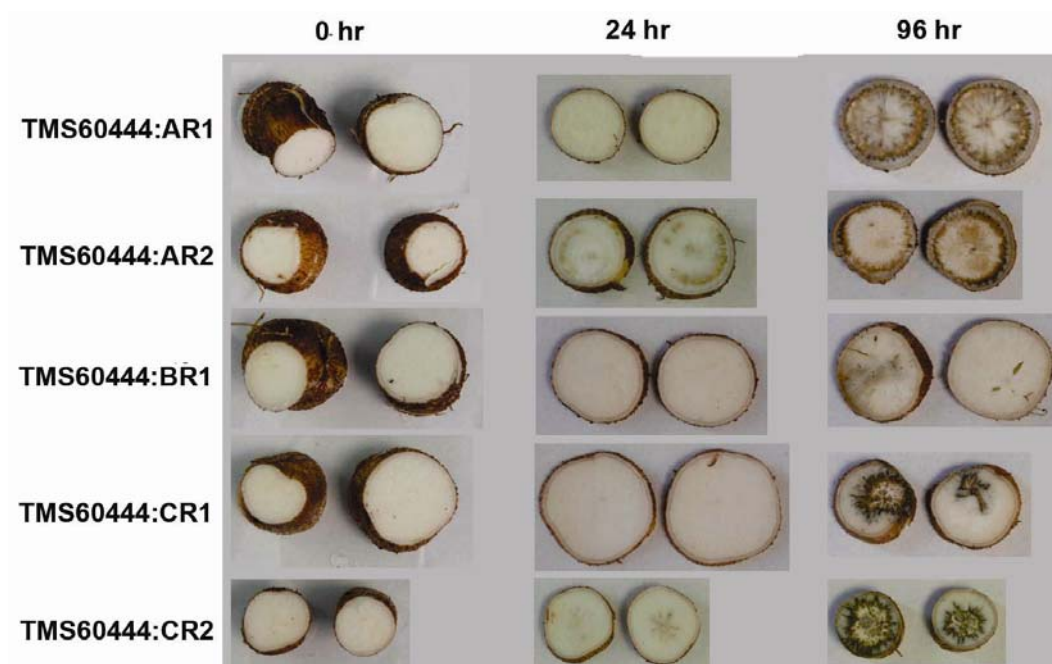


Figure 5.9 Wild-type TMS60444 roots following harvest. Sections of roots shown 0 hr, 24 hr and 96 hr post-harvest. Five roots were harvested and assessed from three plants (TMS60444:A; TMS60444:B and TMS60444:C).

Although symptoms of PPD could be observed, it proved difficult to implement a scoring system (i.e. 0 for symptomless tissue → 5, severe symptoms) due to extensive variation in symptoms. For example, roots APX:DAV:AR2 and APX:DAV:BR1 (96 hr) have severe PPD symptoms and were scored 5, but the symptoms are clearly different despite being from the same plant line (Figure 5.10). Also, almost all roots harvested at 0 hr and 24 hr time points lacked PPD symptoms yet samples were severely deteriorated 96 hr following harvest, suggesting progressive changes in PPD in the storage roots arose between 24 hr and 96 hr post-harvest. Ergo, it was neither possible to measure PPD symptoms nor identify lines that may have delayed PPD based on visual assessments.

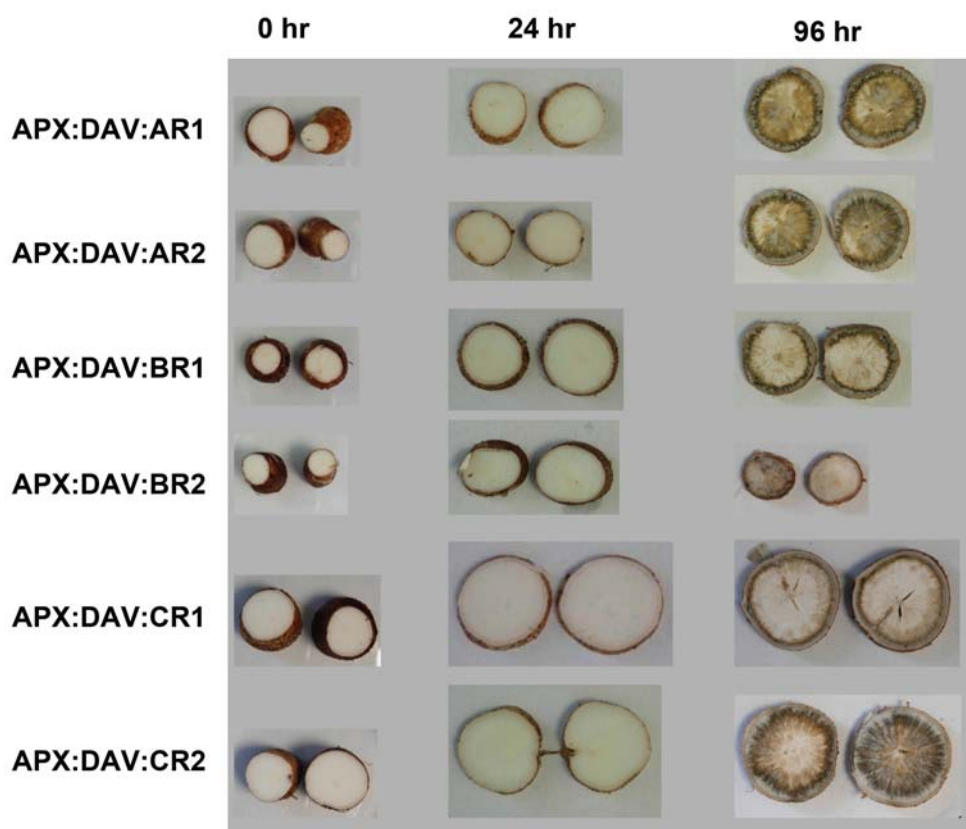


Figure 5.10 Transgenic APX:DAV roots following harvest. Sections of roots shown 0 hr, 24 hr and 96 hr post-harvest. Six roots were harvested and assessed from three plants (APX:DAV:A; APX:DAV:B and APX:DAV:C).

5.3.6 Real-time PCR analysis: amplification primer design and validation of reference genes

Comparative real-time PCR to measure transgene expression requires reference genes whose expression is consistent throughout the differing experimental treatments, tissue and conditions. Real-time PCR is seldom used in cassava research and no reference genes have been characterised for PPD studies. Validation of proposed reference genes was therefore required to permit analysis of transgene expression in the pDEST™-*MecAPX2* plants. Amplification primers for genes *18S*, *SOLUBLE N-ETHYL MALEIMIDE-SENSITIVE FACTOR ATTACHMENT PROTEIN RECEPTORS (SNARE)*, *PROTEIN SERINE/THREONINE PHOSPHATASE (PP2A)*; Figure 5.11), *UBIQUITIN C (UBC)*; Figure 5.12) and *POLYUBIQUITIN 10 (UBQ10)* were designed (Table 2.1) using alignments of published sequence. These genes, whose products are involved in protein synthesis, vesicle trafficking and signalling were selected based on published information

from *Arabidopsis* (Czechowski *et al.*, 2005) and advice (C. Laloi, *pers. comm.*). Amplification primers were designed to anneal at the 3'-end of the coding sequence to maximise amplification efficiency since cDNA was primed using oligo(dT)₂₀ (Section 2.6.4).

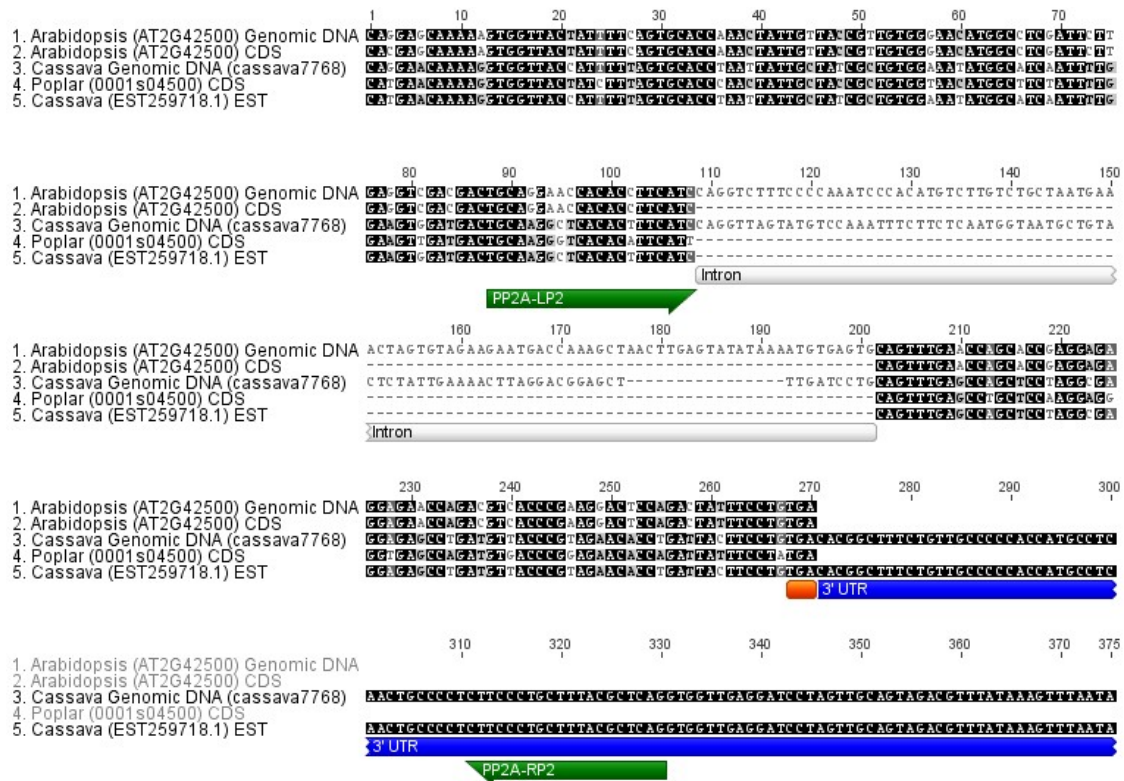


Figure 5.11 Nucleotide alignment of *PP2A* sequence. *Arabidopsis* genomic DNA (TAIR accession AT2G42500; lane 1), *Arabidopsis* coding sequence (TAIR accession AT2G42500; lane 2), cassava genomic DNA (Phytozome database accession cassava7768; lane 3), poplar coding sequence (accession 0001s04500; lane 4) and cassava EST sequence (Cassava Online Database accession EST259718.1; lane 5). Position of intron (grey rectangle), PCR-amplification primers (PP2A-LP2 & PP2A-RP2; green arrows), termination codon (orange box) and 3'-UTR (blue rectangle) shown.

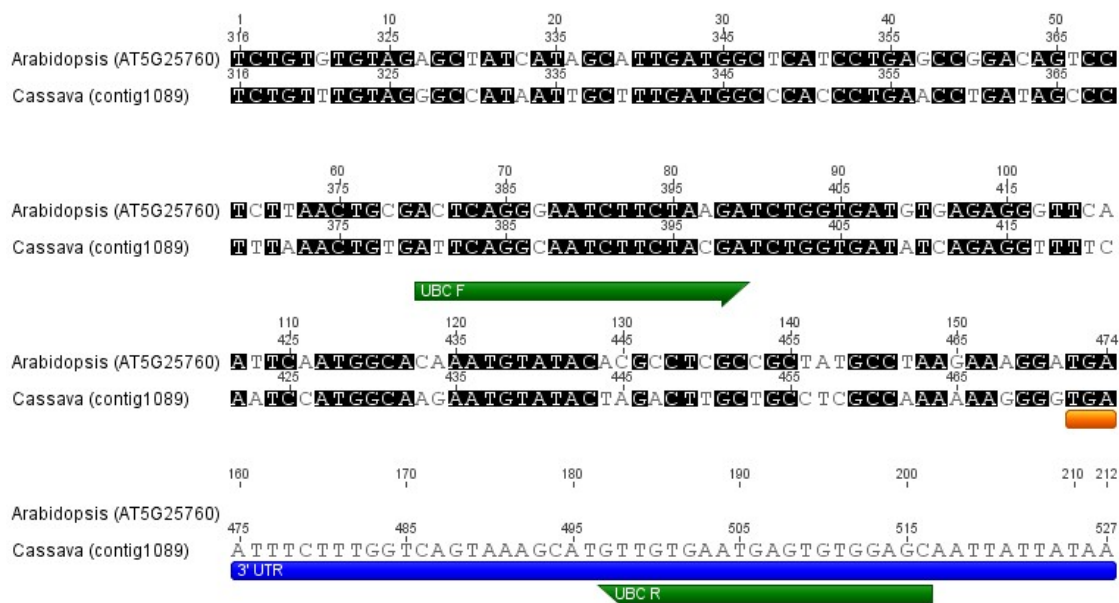


Figure 5.12 Nucleotide alignment of UBC sequence. *Arabidopsis* (TAIR accession AT5G25760) and cassava EST-derived sequence (Cassava Online Database accession contig1089). Positions of PCR-amplification primers (UBC-F and UBC-R; green arrows), termination codon (orange box) and 3'-UTR (blue rectangle) shown.

Primers were also designed for transgene amplification. The sense-strand primer (APX Transgene-F; Table 2.1) flanked an exon-exon boundary to prevent amplification from contaminating genomic DNA, whilst the generic complementary-sense strand primer (Transgene R; Table 2.1) was positioned within the vector (downstream of the termination codon and upstream of the polyA site) to improve amplification specificity (Figure 5.13). Nucleotide BLAST searches revealed the APX Transgene-F sequence aligned to only one position in the cassava genome and no sequence homology to the Transgene R primer was detected.

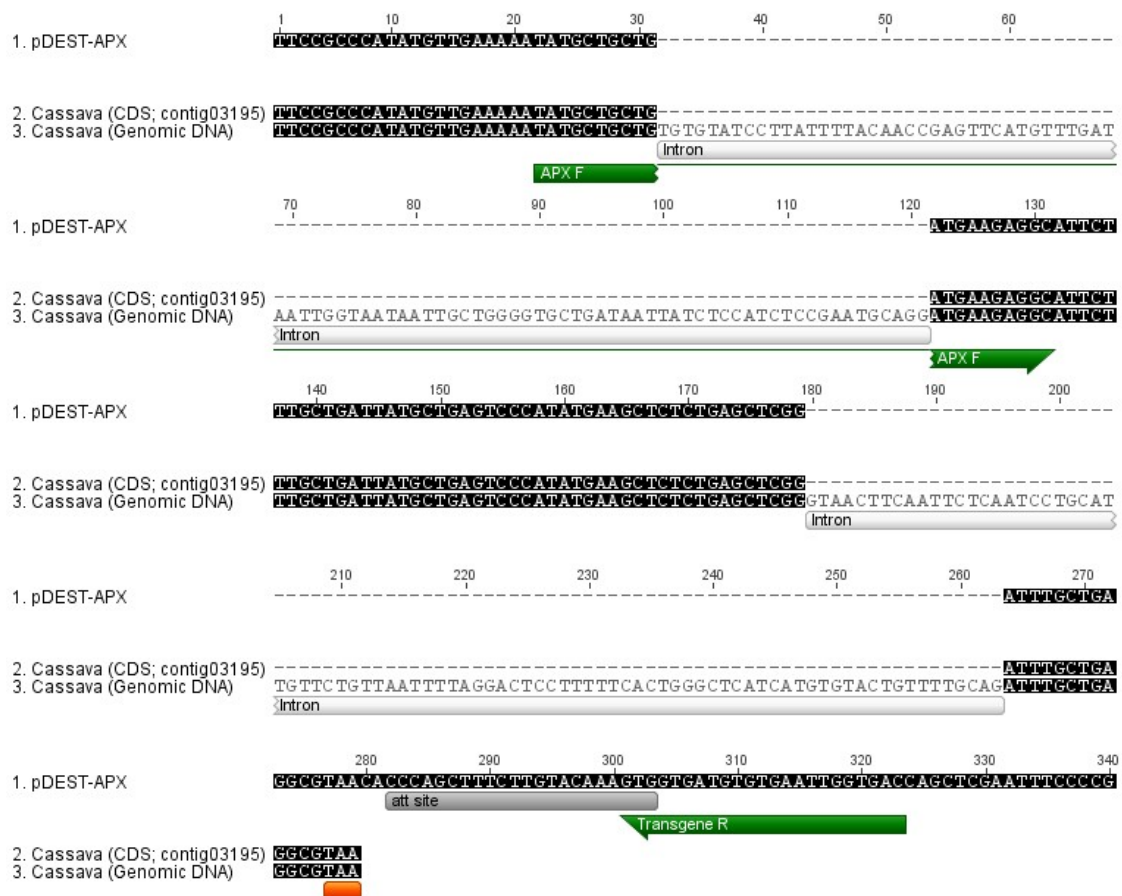


Figure 5.13 Nucleotide sequence alignment of APX. pDEST™-MecAPX2 sequence (lane 1), coding sequence from cassava (Cassava Online Archive accession contig03195; lane 2) and genomic cassava DNA (Phytozome database; lane 3). Positions of introns (grey rectangles), APX Transgene-F & Transgene R primers (green arrows), termination codon (orange box) and Gateway® att-sites (dark grey rectangle) are shown.

5.3.6.1 PCR-amplification of reference genes using *Taq* DNA Polymerase

Amplification primers for the proposed reference genes were used in PCR with standard *Taq* DNA Polymerase (Section 2.1.2) to check specificity. Template cDNA was derived from 1 µg total RNA of wild-type TMS60444:AR1, TMS60444:BR1 and transgenic APX:BG and APX:DAE roots (Sections 2.6.1-2.6.4). Primers for *UBC* (UBC-F & UBC-R) and *SNARE* (SNARE For & SNARE Rev; Table 2.1) generated the predicted sized amplicons (137 bp and 156 bp in length, respectively) observed following agarose gel electrophoresis (Section 2.4.2). Importantly, non-specific priming was not observed for either primer pair, indicating good specificity to the target sequence (Figure 5.14).

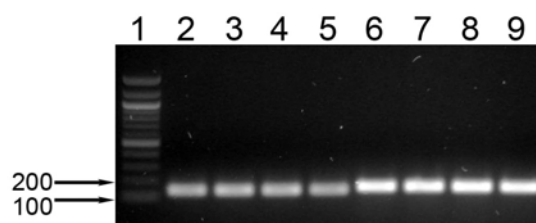


Figure 5.14 Putative *UBC* and *SNARE* PCR-amplification products. *UBC* amplification (lanes 2-5) and *SNARE* amplification products (lanes 6-9) from cDNA derived from wild-type TMS60444:AR1 (lanes 2 & 6), TMS60444:BR1 (lanes 3 & 7), APX:BG:AR1 (lanes 4 & 8) and APX:DAE:AR1 (lanes 5 & 9). DNA ladder (bp) shown (lane 1).

Similar results were obtained following PCR-amplification using primers for *18S* (18S For & 18S Rev; Table 2.1), yielding a single sized product of approximately 169 bp (Figure 5.15a). However, the designed *UBQ10* primers (*UBQ10-F* & *UBQ10-R*) generated various sized products, suggesting sub-optimal amplification specificity (Figure 5.15a). Amplification of *PP2A* sequence was successful, generating a single product of approximately 150 bp (Figure 5.15b) from cDNA, whilst from wild-type genomic DNA the expected larger-sized (227 bp) product (due to the intron) was also produced (Figure 5.15b). Based on these studies all reference gene primer pairs are suitable for real-time PCR analysis with the exception of *UBQ10-F* and *UBQ10-R*.

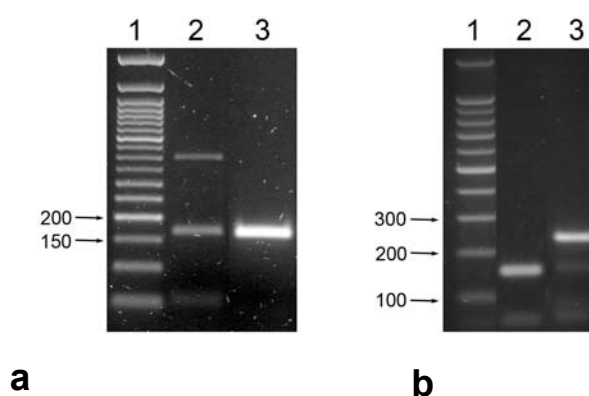


Figure 5.15 Putative *UBQ10*, *18S* and *PP2A* PCR-amplification products. *UBQ10* amplification (lane 2) and *18S* amplification products (lane 3) from cDNA derived from TMS60444:AR1 (**a**). *PP2A* amplification products using template cDNA derived from TMS60444:AR1 (lane 2) and TMS60444 genomic DNA (lane 3) (**b**). DNA ladder (bp) shown (lanes 1).

5.3.6.2 Verification of reference and transgene primers in real-time PCR

Amplification primers for the proposed reference genes (*SNARE*, *UBC*, *18S* and *PP2A*) successfully amplified target sequence from cDNA derived from wild-type TMS60444:AR1 (0 hr and 24 hr post-harvest) in real-time PCR. Five dilutions of cDNA were amplified in duplicate (Section 2.6.4) to generate data for standard curve analysis and melting and amplification profiles. For all primer pairs statistically significant R^2 values ($R^2 > 0.980$) were obtained (Figure 5.16) from the standard curves, indicating minimal variability between assay replicates and that amplification is comparable even with varying quantity of template cDNA. Furthermore, there is only minimal variation in C_T values in amplifications using tissue analysed at 0 hr and 24 hr post-harvest (Figure 5.16). The mean difference in C_T values between the two time points using *18S* primers was a mere 0.20, whilst for *PP2A* primers the difference ranged from 0.20 to 1.02, suggesting stable gene expression even after 24 hr deterioration. Greater variation was observed with *UBC* data, with differences in C_T values ranging from 1.24 to 4.68; the greatest variation arising in the least dilute cDNA samples. No data is available for performance of the *SNARE* primers using material 24 hr post harvest. Importantly, APX Transgene-F and Transgene R primers also amplified successfully ($R^2 = 0.9954$; Figure 5.17) from cDNA derived from APX:BG:AR1 (amplicon 128 bp in length) and, as expected, failed to amplify from TMS60444:AR1. Amplification and melting profiles for all proposed reference genes and transgene were in-keeping with successful and specific amplification (Figures 5.18-5.20). A limited screen of cDNA derived from APX:BG:AR1 was also used to assess primer efficiencies of the reference genes to confirm there was no/minimal variation between tissue (data not shown).

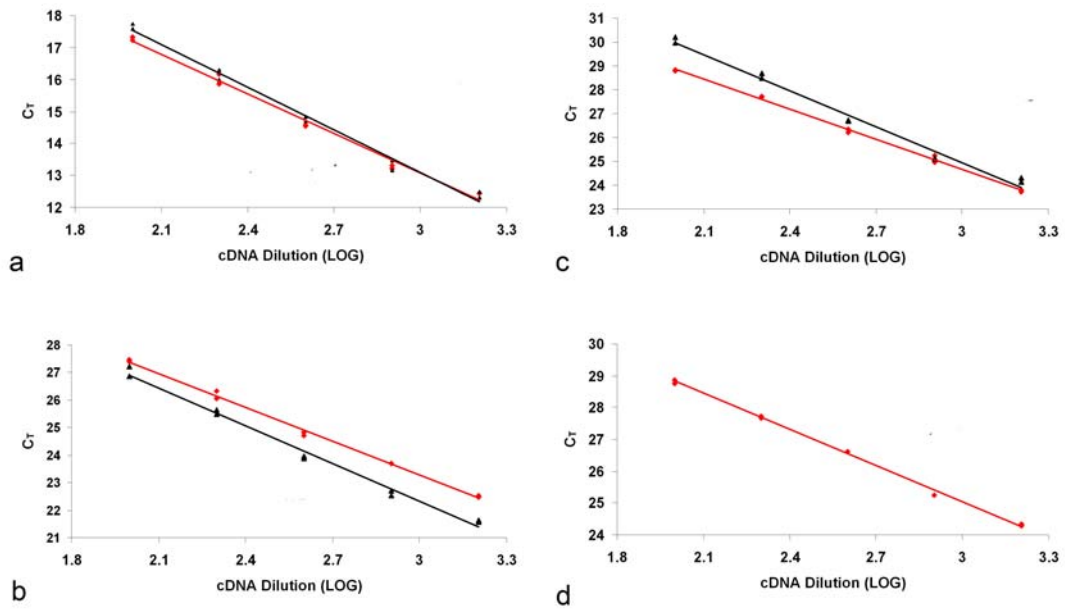


Figure 5.16 Standard curve analysis of real-time PCR amplification. Dilutions of wild-type TMS60444:AR1 derived cDNA amplified with: 18S For & 18S Rev (0 hr: $y = -4.1358x + 25.474$, $R^2 = 0.9904$; 24 hr: $y = -4.4381x + 26.407$, $R^2 = 0.9900$) (a); UBC-F & UBC-R (0 hr: $y = -4.0959x + 35.573$, $R^2 = 0.9967$; 24 hr: $y = -4.5859x + 36.078$, $R^2 = 0.9902$) (b); PP2A-LP2 & PP2A-RP2 (0 hr: $y = -4.2188x + 37.305$, $R^2 = 0.9972$; 24 hr: $y = -5.0311x + 40.032$, $R^2 = 0.987$) (c); SNARE For & SNARE Rev (0 hr: $y = -3.8019x + 36.429$, $R^2 = 0.9973$; 24 hr data not available) (d). 0 hr (red) and 24 hr (black) data points and linear regression lines.

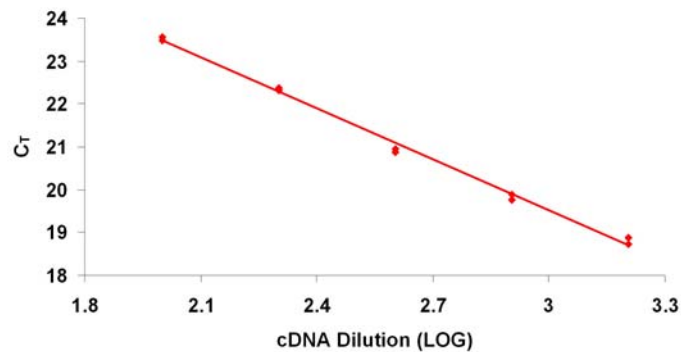


Figure 5.17 Standard curve analysis of PCR amplification using transgene specific primers. Dilutions of APX:BG:AR1 derived cDNA and transgene primers (APX Transgene-F & Transgene R). 0 hr: $y = -3.9747x + 31.422$, $R^2 = 0.9954$; 24 hr: data not available.

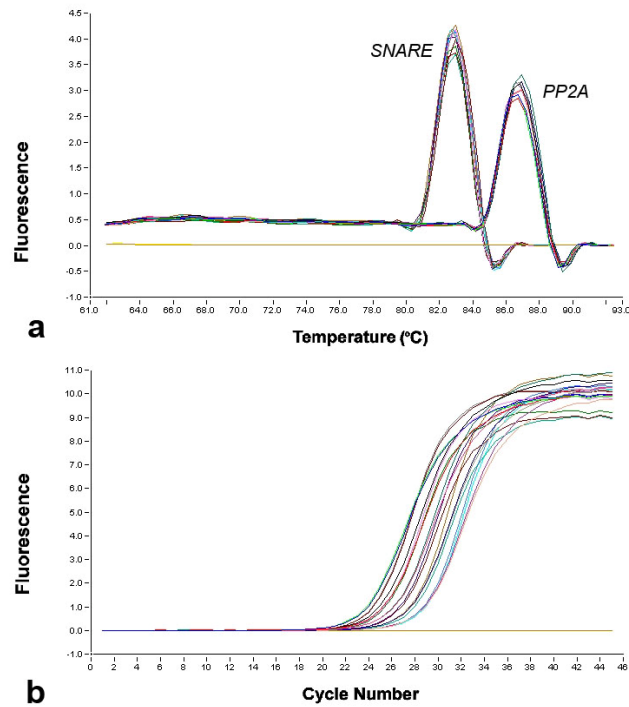


Figure 5.18 Real-time PCR analysis of *SNARE* and *PP2A* amplification. Melting (a) and amplification profiles (b). Amplification from cDNA derived from APX:BG:AR1 material, 0 hr post-harvest.

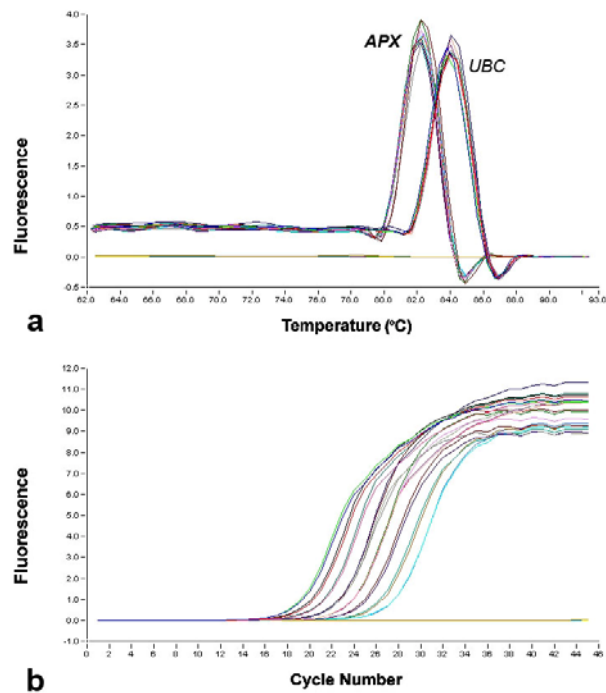


Figure 5.19 Real-time PCR analysis of *APX* and *UBC* amplification. Melting (a) and amplification profiles (b). Amplification from cDNA derived from APX:BG:AR1 material, 0 hr post-harvest.

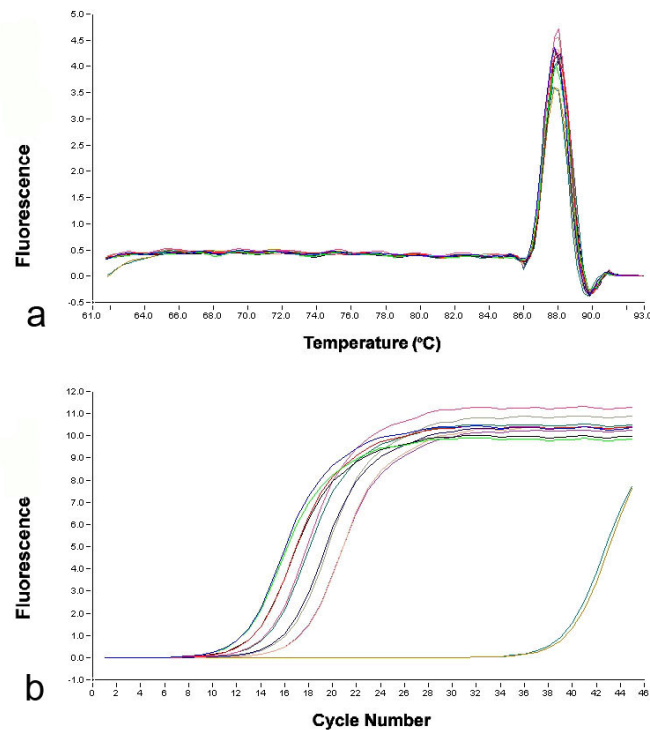


Figure 5.20 Real-time PCR analysis of 18S amplification. Melting (a) and amplification profiles (b). Amplification from TMS60444 wild-type root material, 0 hr post-harvest.

Amplification efficiency (E) was calculated from the slope of the standard curves (Section 2.1.3; Figures 5.16 and 5.17) and varied between primers and age of tissue used (Table 5.3). *PP2A* primers were the least efficient whereas *SNARE* primers (using 0 hr tissue) were the most efficient. Interestingly, all amplification efficiencies were less than the recommended 90-105% (Bio-Rad, 2010). However, although efforts were made to obtain high quality RNA, the exudation of polysaccharides, for example, that are abundant in roots probably influenced primer annealing and amplification efficiencies. This problem was exacerbated through the use of degraded tissue that is high in phenolic compounds, resulting in a 10.4% mean reduction in amplification efficiency between material harvested at 0 hr and 24 hr - an unavoidable consequence of PPD studies.

Table 5.3 Efficiency of real-time PCR amplification.

Gene	Slope* (0 hr)	E (%) 0 hr	Slope* (24 hr)	E (%) 24 hr
<i>18S</i>	-4.1358	74.4	-4.4381	68.0
<i>UBC</i>	-4.0959	75.4	-4.5859	65.2
<i>PP2A</i>	-4.2188	72.6	-5.0311	58.0
<i>SNARE</i>	-3.8019	83.2	n/d	n/d
<i>MecAPX2</i> transgene	-3.9747	78.48	n/d	n/d

* from linear regression calculations (Figures 5.16 and 5.17).

n/d not done

5.3.6.3 Relative efficiencies of reference and transgene primers

The relative efficiencies of proposed reference and target gene primers (ΔC_T comparison; Section 2.1.3) resulted in linear regression slopes of 0.2442 (APX Transgene-F/Transgene R & PP2A-LP2/PP2A-RP2; Figure 5.21a), -0.1727 (APX Transgene-F/Transgene R & SNARE For/SNARE Rev; Figure 5.21b), 0.1213 (APX Transgene-F/Transgene R & UBC-F/UBC-R; Figure 5.21c) and 0.1611 (APX Transgene-F/Transgene R & 18S For/18S Rev; Figure 5.21d), which compare to the recommended slope = 0.1. During the ongoing collection of data *PP2A* was selected as the reference gene to measure transgene expression in pDEST™-*MecAPX2* transformed lines.

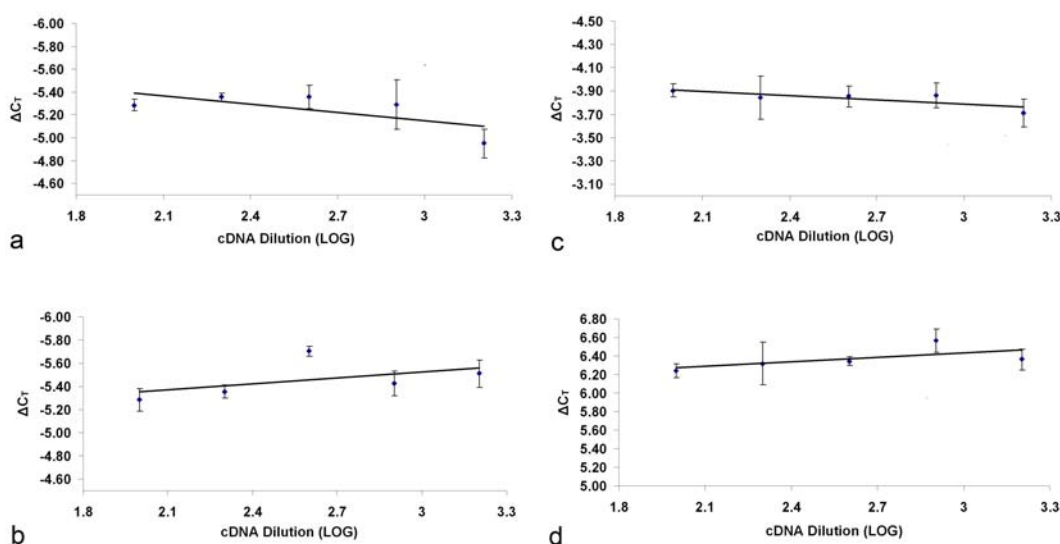


Figure 5.21 Relative efficiency of reference and transgene amplification. APX Transgene-F/Transgene R & PP2A-LP2/PP2A-RP2 ($y=0.2442x-5.8823$, $R^2 = 0.4705$) (a); APX Transgene-

F/Transgene R & SNARE For/SNARE Rev ($y=-0.1727x-5.0065$, $R^2 = 0.2567$) **(b)**; APX Transgene-F/Transgene R & UBC-F/UBC-R ($y=0.1213x-4.1505$, $R^2 = 0.6097$) **(c)**; APX Transgene-F/Transgene R & 18S For/18S Rev ($y=0.1611x+5.9488$, $R^2 = 0.4143$) **(d)**. S.D. shown.

5.3.7 Comparative analysis of *MecAPX2* expression in transgenic cassava

Two or three storage roots from each of the eight independent lines of pDEST™-*MecAPX2* transformed cassava were selected and used for real-time PCR analysis following RNA extraction (Figure 5.22) and RT-PCR (Section 2.6). Expression of the transgene was calculated relative to the *PP2A* reference gene using the formula $2^{-\Delta CT}$ (Section 2.1.3) and due to time constraints only material sampled 0 hr post-harvest was analysed.

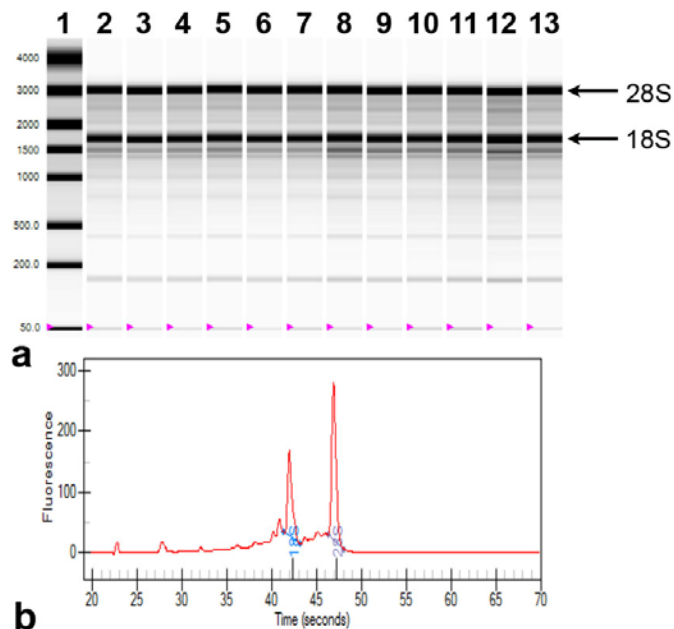


Figure 5.22 Analysis of RNA extracted from cassava roots. Selected samples - APX:CT:AR1 (lane 2), APX:CT:BR1 (lane 3), APX:CT:CR1 (lane 4), APX:CX:AR1 (lane 5), APX:CX:BR1 (lane 6), APX:CX:CR1 (lane 7), APX:DAE:AR1 (lane 8), APX:DAM:AR1 (lane 9), APX:DAM:BR1 (lane 10), TMS60444:AR1 (lane 11), TMS60444:BR1 (lane 12), TMS60444:CR1 (lane 13) and RNA ladder (lane 1). All samples 0 hr post-harvest **(a)**. Chromatogram of APX:CT:AR1 **(b)**. Data obtained using the Experion™ Automated Electrophoresis System.

Interestingly, transgene expression could not be detected in any root of line APX:DAV (APX:DAV:AR2, APX:DAV:BR1 or APX:DAV:CR1), with relative expression and S.D. calculated at 0.00074, 0.12; 0.00099, 0.30 and 0.03461, 0.35, respectively (Figure 5.23). Further investigation is required but this result may simply reflect an experimental error. Variation in relative expression between roots from the same line was also apparent highlighting the need for more biological replicates. For example, roots APX:BG:AR1, APX:BG:BR1 and APX:BG:DR1 recorded relative expression and S.D. values of 18.54, 0.39; 76.10, 0.07 and 46.37, 0.09, respectively. Importantly, APX Transgene-F and Transgene R primers that are specific to the pDEST™-MecAPX2 construct failed to amplify a product from wild-type roots (TMS60444:AR1, TMS60444:BR1 and TMS60444:CR1), in-keeping with expectations.

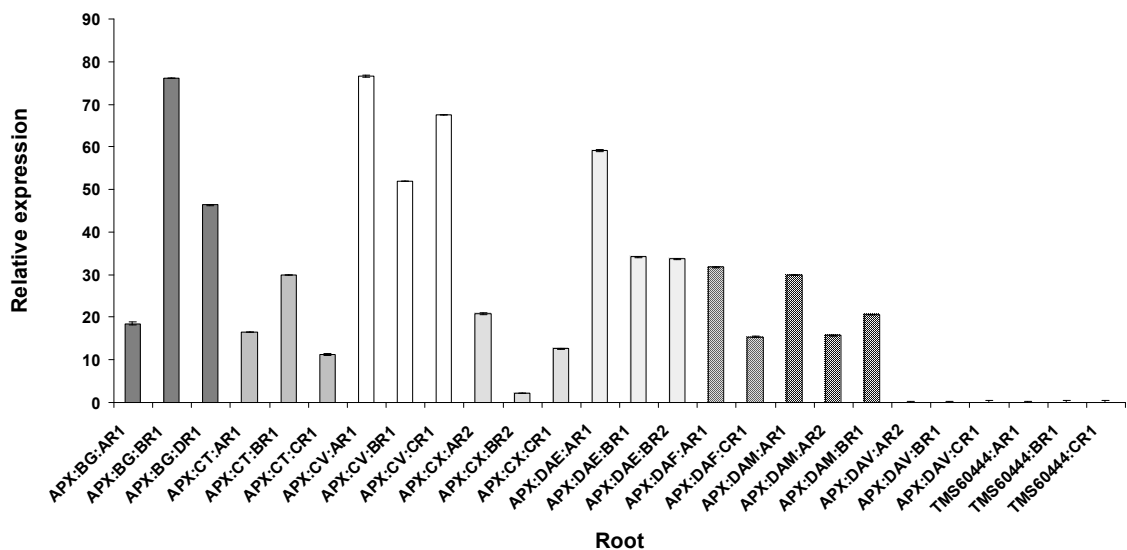


Figure 5.23 Expression of APX transgene compared to PP2A at 0 hr post-harvest. Three storage roots analysed per line, except for APX:DAF where two roots were analysed. Wild-type roots (TMS60444:AR1, TMS60444:BR1 and TMS60444:CR1) and S.D. shown.

Interestingly, despite previous reports suggesting the *St*PAT promoter is root specific in transgenic cassava (Ihemere *et al.*, 2006), data here clearly reveals over-expression of the transgene in leaf tissue of all transgenic lines (Figure 5.24). Indeed, the mean relative expression in leaves for all lines (excluding APX:DAV where leaf data was not collected) is 2.87-fold greater than in the roots. Overall, line APX:CV had the greatest expression of the transgene in both roots and leaves (Figure 5.24).

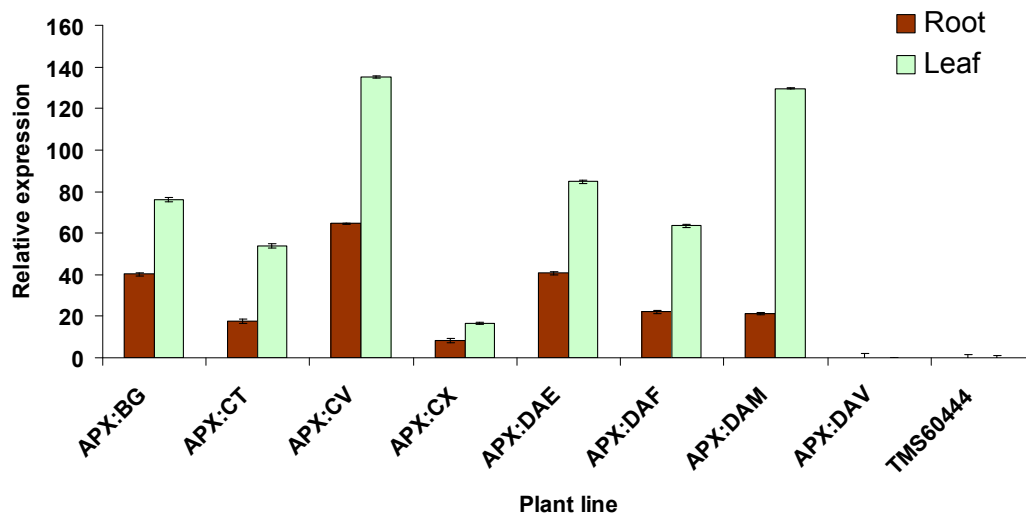


Figure 5.24 Mean expression of APX transgene compared to PP2A in roots and leaves of independent lines. Data (0 hr post-harvest) from eight independent plant lines of pDEST™-MecAPX2 transformed plants. Wild-type data (TMS60444) and S.D. shown. Note: leaf data for APX:DAV not collected.

5.3.8 APX enzyme activity

In addition to transgene expression analysis, preliminary data was collected for APX activity. Protein extracts from root samples (0 hr post-harvest) APX:BG:AR1, APX:CT:AR1, APX:CV:AR1, APX:CX:AR1, APX:DAE:AR1, APX:DAF:AR1, APX:DAM:AR1, APX:DAV:AR2 representing each of the eight lines and wild-type TMS60444:AR1 were prepared and used to measure APX activity (Sections 2.7.1-2.7.3). Due to time constraints it was not feasible to analyse additional biological samples. Activity of APX in lines APX:BG and APX:CV were commensurate with transcript analysis, representing both increased transcription and activity (Figure 5.25). Additionally, minimal activity was recorded in line APX:DAM (Figure 5.25) that also had comparatively low expression of the transgene as measured by real-time PCR. Most surprising is the significant level of APX activity in line APX:DAV that was 4-fold higher than APX:BG, the line with the second highest level of activity, despite no transgene transcript/amplification being detected in real-time PCR analysis (Figure 5.25).

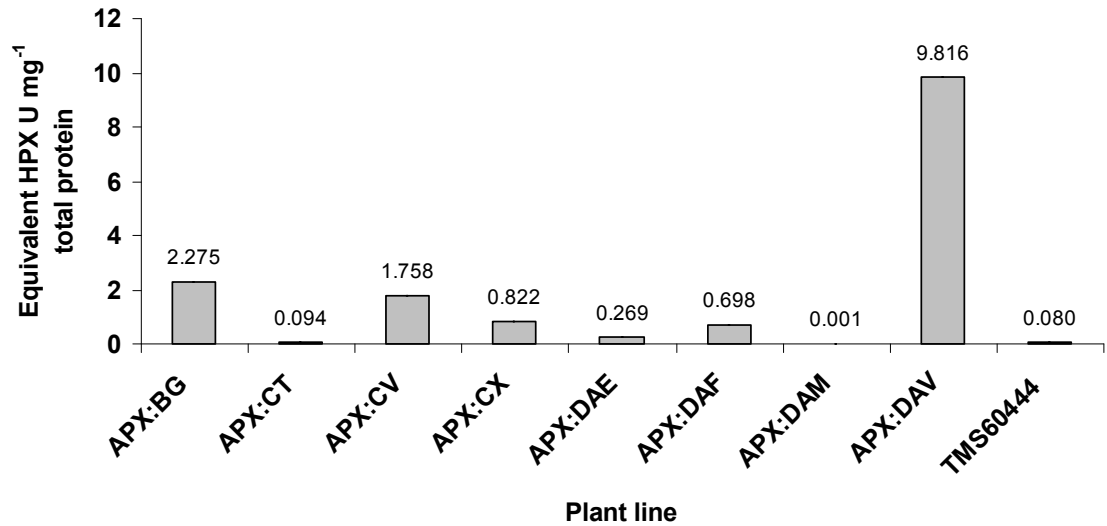


Figure 5.25 Mean equivalent HPX units (APX activity) in pDEST™-*MecAPX2* transformed and wild-type roots. Eight pDEST™-*MecAPX2* transgenic lines and wild-type tissue (TMS60444). For clarity values are shown per line ($n=2$), S.E. shown.

A preliminary screen of enzyme activity using field-grown roots of three cultivars with varying susceptibility to PPD revealed an increase in APX activity following harvest (Figure 5.26). This increase is most dramatic in TMS60444 tissue compared with cultivar MVEN-77, which is reportedly less susceptible to PPD. These data provide useful baseline information into the characteristics of cultivar TMS60444 and it will be interesting to analyse and compare the glasshouse-cultivated transgenic roots at the later time points (i.e. 24 hr and 96 hr post-harvest) to determine whether this activity profile is conserved.

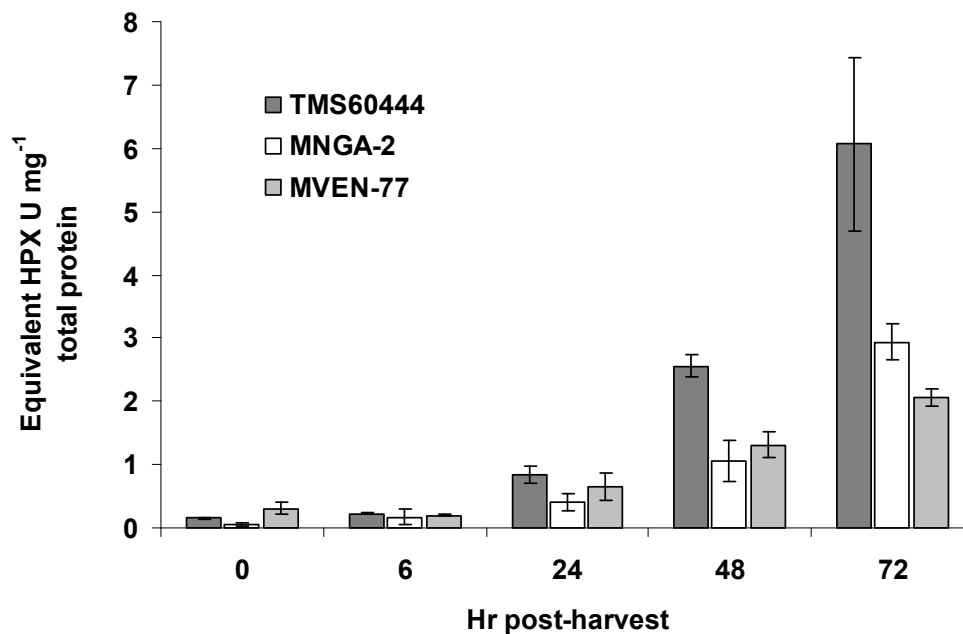


Figure 5.26 Mean HPX equivalent units (APX activity) in cultivars of cassava with varying susceptibility to PPD. TMS60444 (most susceptible), MNGA-2 (moderately susceptible) and MVEN-77 (least susceptible). $n=2$ and S.E. shown.

5.4 DISCUSSION

5.4.1 Successful production of pDEST™-MecAPX2 transgenic cassava

This chapter describes the production of more than 150 *in vitro* pDEST™-MecAPX2 plantlets, which was made possible following improvements to the *Agrobacterium*-mediated transformation protocol (discussed in Chapter 4). Approximately 44 plants with storage roots suitable for analysis were produced in approximately five months in the glasshouse at the University of Bath. No phenotypic abnormalities were observed (e.g. stunted growth and leaf discolouration) and there was no statistical difference (determined by independent-samples *t*-tests) in either plant height or root stock weight of the transgenic material compared with wild-type plants. It is interesting to note that more than 10 independent lines were identified from only 30 analysed plants, whereas only approximately five lines of cassava were generated by Chellappan *et al.* (2004), Ihemere *et al.* (2006) and Vanderschuren *et al.* (2007).

5.4.2 High proportion of single hybridised fragments amongst transgenic plants

It was unexpected that all tested transgenic plants had only one (77% of total) or two (23% of total) insertion fragments (i.e. genomic fragments to which the DIG-*hptII* probe annealed). Publications by Ihemere *et al.* (2006), Schreuder *et al.* (2001) and Zhang *et al.* (2003b) reported single genomic fragment hybridisations in only 33%, 18% and 42%, respectively, of plants. The reason for this phenomenon is unknown but perhaps integration capacity was influenced by the incorporation of Gateway[®] infrastructure in the expression cassette. Alternatively, were modifications to the transformation protocol important? For example, co-culturing *Agrobacterium* and FEC directly on culture plates, exclusion of liquid media stages or recovery phase following co-cultivation? An important consideration is whether embryos/plantlets with T-DNA in numerous genomic locations were incapable of regeneration and/or phenotypically abnormal and thus not selected for further growth. Multiple copies of a transgene have been reported to result in gene silencing (Vaucheret *et al.*, 1998) and it is therefore possible that, should APX be silenced in the transgenic plantlets, then accumulation of H₂O₂ may affect regeneration capacity or phenotype. Presumably, however, endogenous expression of genes such as CAT would be up-regulated to maintain homeostasis. In addition, the inability to PCR amplify the transgene or *hptII* from some plant samples may be associated with aberrant T-DNA insertions missing one or both ends. This situation has been described in transgenic tobacco (Gheysen *et al.*, 1990) and more extensive analysis of the transgenic cassava plants is required to assess the extent of T-DNA incorporation, transgene copy number and vector backbone integration in the plant genome. Importantly, adoption of the improved cassava transformation protocol made available a significant number of plantlets for screening.

5.4.3 Scoring of PPD in harvested glasshouse-cultivated storage roots is complex

The trial experiments to determine an appropriate technique to assess PPD in glasshouse-cultivated storage roots - a practice for which there are no published reports - resulted in the use of partially intact roots. This induced symptoms typical of PPD but unfortunately the analysis proved inconclusive, in part because of the selected time points. Generally, no symptoms were observed at 24 hr post-harvest but by 96 hr post-harvest all samples had severe PPD symptoms, rendering comparative analysis impossible. A late time point of 48 hr or possibly 72 hr post-harvest may have aided a clearer visual distinction between lines. However, it should be considered that whilst a 96

hr time point may not have been appropriate for lines described in this chapter, it cannot be ruled out that distinguishable differences may be observed in plants transformed with other constructs. Thus, it is likely that preliminary trials will need to be undertaken prior to harvest in future experiments to ensure appropriate time points are selected.

In addition to the challenges faced in determining appropriate time points, the significant variation in symptoms, not only between roots from the same line but even between roots from an individual plant, also hindered interpretation. There is no clear explanation for symptom variation, but it probably reflects different stages of root development. Introducing more time points would enable an all-embracing analysis of symptoms, but as experienced when undertaking the assay trials, the large sample sizes, time and labour required is simply too demanding upon resources. One solution could be to use a greater number of time points but simply fewer roots. It may also be productive to grow more plants per line and use the “whole root method” on only the largest root of each plant. This would certainly require more resources but may lead to measurable data that is sufficient to identify key lines. A recent report by Salcedo *et al.* (2010) highlights the difficulties also faced with assessing PPD in field-grown roots. They showed there was no correlation between the accumulation of hydroxycoumarins (measuring fluorescence using image analysis software) and visual assessment, suggesting the former is not a reliable technique and visual assessment, although susceptible to personal interpretation, remains the preferred approach. Clearly, significant progress has been made with regard to PPD assays using glasshouse-cultivated storage roots and further optimisation should eventually result in establishment of a common protocol.

5.4.4 New data validating reference genes for real-time PCR

This is the first report of real-time PCR analysis of cassava roots undergoing PPD and thus provides original information pertaining to the validation of suitable reference genes. Real-time PCR is largely unused in cassava research, possibly because no baseline data has been published and also perhaps due to the intrinsic problems encountered with generating transgenic cassava for analysis. However, progress is being made and recently *18S* has been used as the reference gene in promoter expression analysis (Beltrán *et al.*, 2010) and analysis of plants with increased production of provitamin A (Welsch *et al.*, 2010). *18S* was also used for PPD time course analyses, albeit in Northern blot hybridisation (Reilly *et al.*, 2007). However, due to the possibility of biological variability in samples and fluctuations in gene expression, Bustin *et al.* (2009)

highly recommends that more than one reference gene is used for real-time PCR analysis. Data in this chapter provides evidence that *18S* as well as *PP2A*, *SNARE* and *UBC* are all potentially suitable candidates for comparative gene analysis. *UBQ10* may also be appropriate but requires further investigation. Validation is required for all proposed reference genes using material from later time points e.g. 48 hr, 72 hr and 96 hr post-harvest, which unfortunately was not feasible to complete as part of this investigation. Elucidating a set of reference genes is an important task and similar ventures are being undertaken, predominately in *Arabidopsis* (Czechowski *et al.*, 2005) but also in other plant species, such as poplar (Brunner *et al.*, 2004; Regier & Frey, 2010).

The PCR amplification efficiencies (*E*) of the reference and target genes are lower than the generally recommended 90-105% (Bio-Rad, 2010), suggesting PCR conditions are sub-optimal. However, Schmittgen & Livak (2008) state that there are no particular rules regarding the tolerance margins of *E* and that amplifications of target and reference sequence should be within 10%; a criteria that is fulfilled in this chapter. Whilst the RNA extraction procedure used in this investigation is sufficient to provide good quality RNA there may be contaminants that impede amplification. This explanation is substantiated by the fact that *E* reduces when PCR is used with more deteriorated samples from 24 hr time point – an unavoidable aspect of studying PPD in cassava roots. Although the tissue may present unwelcome complications, endeavours were made to minimise further variability *via* the use of Experion™ RNA analysis chips, which provide a highly advanced and accurate method to determine RNA quantity and integrity. Furthermore, a 100-fold difference in calculated gene expression can occur depending on the choice of RT enzyme used (Ståhlberg *et al.*, 2004) and thus the most efficient SuperScript™ III (Invitrogen) was used to prepare cDNA. Optimisation of real-time PCR techniques should be considered in future investigations but it is apparent that using deteriorated cassava roots will almost inevitably affect *E*.

5.4.5 Experiment design affects real-time PCR data interpretation

Real-time PCR data was analysed using the $2^{-\Delta CT}$ method, a simplified version of the $2^{-\Delta\Delta CT}$ described by Livak & Schmittgen (2001) and Schmittgen & Livak (2008). There are various techniques that can be used for gene expression analysis depending upon experiment design (Bustin *et al.*, 2009), including a formula described by Pfaffl (2001):

$$\text{Expression ratio} = \frac{(E_{\text{target}})^{\Delta C_T \text{ target (calibrator - test)}}}{(E_{\text{ref}})^{\Delta C_T \text{ ref (calibrator - test)}}$$

Where E_{target} is the amplification efficiency of the target gene; E_{ref} amplification efficiency of the reference gene; $\Delta C_T \text{ target (calibrator - test)}$ is the C_T of the target gene in the calibrator minus the C_T of the target gene in the test sample; $\Delta C_T \text{ ref (calibrator - test)}$ is the C_T of the reference gene in the calibrator minus C_T of the reference gene in the test sample.

The $2^{-\Delta C_T}$ formula assumes equal E for target and reference gene, whereas the Pfaffl formula incorporates E and thus provides a more accurate expression ratio. Given that data presented in this chapter reveals a decrease in E using material sampled at 24 hr post-harvest, it would be interesting to apply this formula in future experiments. It was not possible to adopt for this investigation since the formula also requires data from a calibrator, in this scenario wild-type tissue, and thus requires primers to amplify the “gene-of-interest” (GOI) rather than the transgene specifically. The data presented in this chapter clearly demonstrates successful expression of the transgene in most transgenic lines, indicating construct stability *in planta*.

5.4.6 StPAT promoter ostensibly regulates transgene expression in both roots and leaves of cassava

The real-time PCR data in pDEST™-MecAPX2 lines surprisingly revealed expression in leaves. This was unexpected since research by Ihemere *et al.* (2006) reported the StPAT promoter to be root specific in cassava. Further experiments are required to understand this result but it is conceivable that the close proximity (approximately 250 bp) of the CaMV35S promoter regulating *hptII* expression to the StPAT promoter may be resulting in constitutive expression of the transgene. An elegant study by Yoo *et al.* (2005) used constructs containing the promoter of *LATERAL ROOT PRIMORDIA1*, a root-specific gene, revealing *trans* activation of the transgene when the CaMV35S promoter was also incorporated into the construct. This hypothesis would certainly explain the constitutive expression observed in the transgenic lines. Moreover, the construct used by Ihemere *et al.* (2006) arranged genes in series ensuring a greater distance between the promoters, whereas for this investigation the divergent promoters were either side of the MCS and therefore in much closer proximity to one another. Whilst this seems a feasible explanation it is interesting to note that pDEST™-GUSPlus in *Arabidopsis* plants resulted

in *GUS* expression predominately in the roots (Page, 2009; Figure 5.27). Critically, the transgenic cassava plants harbouring the pDEST™-*GUSPlus* and pDEST™-*GUSPlus*(-)PAT will provide important information regarding gene expression in cassava and which are currently being grown in the glasshouse (Chapter 7).

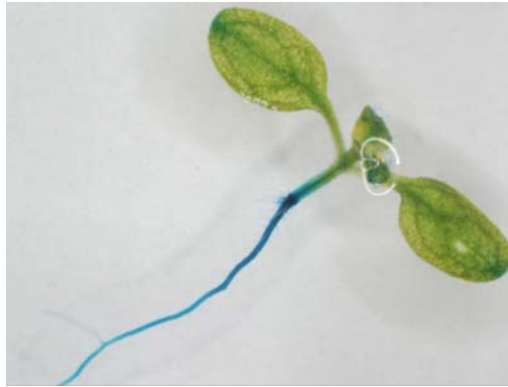


Figure 5.27 GUS detection in pDEST™-*GUSPlus* transformed *Arabidopsis*. Expression clearly visible predominately in root tissue. Figure courtesy of M. T. Page (University of Bath).

The *StPAT* promoter is probably derived from the *StB33* promoter (Rocha-Sosa *et al.*, 1989) but lacks approximately 45 bp immediately upstream of the transcriptional start site. The 22 bp sequence that is used predominately to classify *PATATIN* promoters as class I or class II (Pikaard *et al.*, 1987) lies within this missing region and thus, whilst *StPAT* sequence is highly homologous to other class I *PATATIN* promoters the absence of this region prevents unambiguous classification of *StPAT* as class I (i.e. predominantly tuber specific). *StPAT* was used by Ihemere *et al.* (2006) and therefore, despite the anomalous sequence, has been proven to be root specific in transgenic cassava. Recently, promoter deletion analysis has revealed the *StPAT* promoter to be wound inducible, with *cis* regulatory motifs located in the 261 bp region at the 3' end. However, no increase in transgene transcript was detected after wounding – a discovery that is purportedly due to the time period between wounding and analysis; increased expression was only transient and returned to basal levels prior to analysis. Alternatively, transcript data may be explained by the sampling strategy i.e. a whole leaf was harvested but only a few cells exhibited a wound response (Page, 2009). These observations or their significance have not been studied in cassava but will be an important consideration with the cassava transformed with pDEST™-*GUSPlus* and pDEST™-*GUSPlus*(-)PAT.

The notable variation in gene expression between root and leaves from the same line probably reflects the challenges faced with scoring and assessing PPD in cassava. More biological samples will of course improve data interpretation and mathematical models can be used to standardise replicates (Willems *et al.*, 2008). Variation in gene expression may be influenced by the developmental stage of the storage root, transgene copy number and/or by the position of T-DNA integration. van Leeuwen *et al.* (2001) used *luc*-transformed *Arabidopsis* to demonstrate that the position of T-DNA affects spatial and temporal promoter activity. The results for line APX:DAV were curious – almost undetectable levels of transcription based on real-time PCR analysis and yet this line appears to have the highest level of enzyme activity. Whilst experimental error cannot be ruled out, post-transcriptional gene silencing may also be possible, with increased activity of endogenous POX to accommodate suppressed *APX* expression.

The increasing level of enzyme activity in TMS60444 between 6 hr and 72 hr has also been reported recently by Owiti *et al.* (2011), with expression increasing from 12 hr to 96 hr. Interestingly, however, this data neither tallies with protein abundance that declines during late PPD nor transcript abundance (J. Owiti, *unpublished data*). Reilly *et al.* (2007) also showed an increase in activity during PPD but peaking at 24 hr post-harvest, unlike either the data present here or by J. Owiti. These various discrepancies could be explained by the different analytical techniques employed; iTRAQ and mRNA analysis were based on a restricted number of isoforms, whereas enzyme activity measures not only *APX* but the substrate is also catalysed by guaiacol peroxidases.

5.4.7 Future work

The results presented in this chapter provide a basis for further and necessary experiments to characterise pDEST™-MecAPX2 transformed plants. These include expansion of real-time PCR to include more biological samples as well as to incorporate material from the late-harvest time points. Not only will these data provide information about *APX* expression but will be coupled with improved characterisation of the proposed reference gene expression profiles. With advances in real-time PCR it would be interesting to review the discrepancy in the *APX* expression profile between data obtained from microarray and Northern blot hybridisation (Reilly *et al.*, 2007). Given that transgene expression has been confirmed in this chapter, it would seem prudent to extend analysis and amplify the GOI (*cAPX*), permitting baseline data collection for wild-type cassava. Other work may also elaborate on enzyme assays to include associated

enzymes, such as CAT, measure the influence of *APX* transgene. Notably, given that the method described in this thesis was labour-intensive, trials have now been initiated to utilise a microplate system that allows easy measurements of APX and associated enzymes (Murshed *et al.*, 2008). If promising results emerge then measuring ascorbate content (Gillespie & Ainsworth, 2007; Vislisel *et al.*, 2007) and perhaps inducing oxidative stress (e.g. high light intensity or MV) could also be considered.

6 CASSAVA TRANSFORMATION WITH γ -GLUTAMYLCYSTEINE SYNTHETASE

6.1 INTRODUCTION

6.1.1 Synthesis of glutathione in plants

The two-step pathway for glutathione synthesis in plants occurs in plastids (in particular the chloroplast) and the cytosol. γ -glutamylcysteine synthetase (γ -GCS; EC 6.3.2.2), also sometimes referred to as glutamate-cysteine ligase, synthesises γ -glutamylcysteine (γ -EC) from L-cysteine and L-glutamate in an ATP-dependent reaction (Figure 6.1). In *Arabidopsis*, γ -GCS is encoded by a single gene (*GSH1*; TAIR accession AT4G23100) and includes transit peptide sequence at the 5'-end that locates the protein exclusively in plastids. This has been convincingly shown *via gfp* and red fluorescent protein (*rfp*) reporter gene fusions and immunocytochemistry in *Arabidopsis* and *Brassica juncea* leaves and suspension cultures (Wachter *et al.*, 2005). *GSH1* in plants was first sequenced from *Arabidopsis* (May & Leaver, 1994) but has since been characterised in other species, including *B. juncea*, *Pisum sativum*, *Picea abies*, *Glycine max*, *R. communis* and *Lycopersicon esculentum* (NCBI database search). Phylogenetic analyses led to the categorisation of three classes of *GSH1*; (1) γ -proteobacteria (e.g. *E. coli*; Watanabe *et al.*, 1986), (2) non-plant eukaryotes (e.g. mammals and *Drosophila*) and (3) plants and α -proteobacteria. Although there is no significant pairwise similarity between groups, conserved amino acid sequence motifs were identified (Copley & Dhillon, 2002).

The intermediate product (γ -EC), which has no discernible function in plants, is converted to reduced glutathione (GSH) with the addition of L-glycine in a reaction catalysed by glutathione synthetase (GSH-S; EC 6.3.2.3; Figure 6.1). GSH-S is also encoded by a single gene in *Arabidopsis* (*GSH2*; TAIR accession AT5G27380), which generates two transcripts - the shorter (more abundant) transcript encodes a cytosolic GSH-S whilst the longer transcript/protein is targeted to plastids (Wachter *et al.*, 2005). Thus, the first stage in glutathione synthesis occurs only in plastids whilst the second reaction can arise either in plastids or the cytosol.

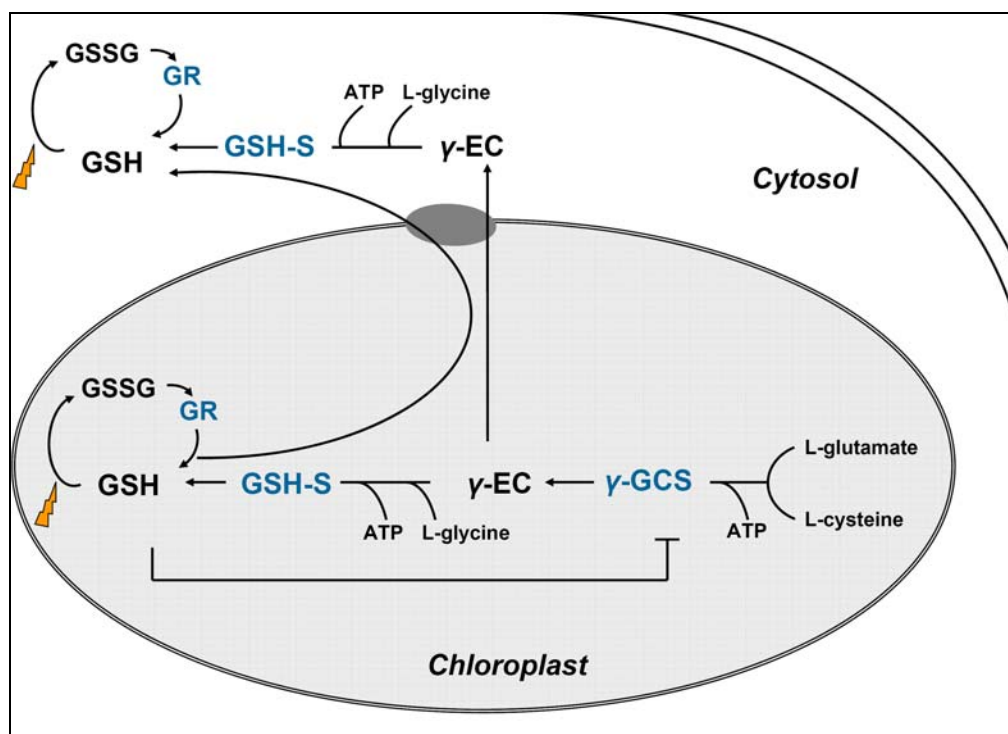


Figure 6.1 Simplified pathway for glutathione synthesis in plants. L-cysteine and L-glutamate are used to produce γ -glutamylcystine (γ -EC) via an ATP-dependent reaction catalysed by γ -glutamylcystine synthetase (γ -GCS). γ -EC can be exported from the chloroplast to the cytosol and is converted to reduced glutathione (GSH) with the addition of L-glycine, a reaction catalysed by glutathione synthetase (GSH-S). GSH synthesised in the chloroplast can be exported to other cellular locations. Oxidation of GSH via ROS and enzymatic reactions (orange arrows) results in GSSG production but which in-turn can be reduced by glutathione reductase (GR). Enzymes in blue font.

6.1.2 Forms and functions of glutathione in plants

Glutathione is a thiol tripeptide (γ -Glu-Cys-Gly; $C_{10}H_{17}O_6N_3S$) and homologous GSH forms have also been identified in some plant species that have substituted L-glycine with other C-terminal amino acids. For example, γ -Glu-Cys- β -Ala (homoglutathione; hGSH; Macnicol, 1987) and γ -Glu-Cys-Ser (hydroxymethylGSH) have been identified in legumes and cereals, respectively (cited in Foyer *et al.*, 2005). The presence of homologous GSH is likely to be the result of gene duplication and divergence in specificity during evolution. The pathway for the production of such alternative forms of GSH is not well understood but research suggests that, in legumes at least, they are synthesised from genes different to those in the GSH pathway (Macnicol, 1987). In the root nodules of the legumes bean (*Phaseolus vulgaris*), mungbean (*Vigna radiata*) and

soybean (*Glycine max*), hGSH was the most abundant tripeptide thiol (Matamoros *et al.*, 1999). In cassava, no alternative forms of GSH have been reported, although current knowledge regarding glutathione and its synthesis in this plant species is remarkably scarce and therefore it is highly unlikely homologous GSH would have been discovered.

Glutathione has prominent roles in plants systems, including responding to biotic and abiotic stress. It is the most abundant form of organic sulphur in plants apart from that incorporated into proteins. Being less reactive than cysteine, glutathione is crucial for sulphur storage and transport *via* the phloem. It is also important in the detoxification of heavy metals, such as cadmium and copper, serving as a precursor of phytochelatins ((γ -Glu-Cys)_n-Gly) that bind and sequester such metals to the vacuole. Other functions include the detoxification of xenobiotics *via* the conjugation with GSH and catalysed by glutathione transferases (GSTs); the resulting complexes are subsequently transported to the vacuole (Dixon *et al.*, 1998). Finally, glutathione has also been implicated in drought and UV protection with relevance to signalling and regulatory pathways (Ball *et al.*, 2004; Gomez *et al.*, 2004; Meyer, 2008). These various functions have been comprehensively reviewed (Foyer *et al.*, 2005; Maughan & Foyer, 2006; Szalai *et al.*, 2009) and it is beyond the scope and objectives of this chapter to discuss them all in detail. Specifically, it is the role of glutathione in the detoxification of H₂O₂ that is the focus for this study into cassava PPD.

6.1.3 Glutathione and its role in H₂O₂ detoxification

The multitude of roles of glutathione necessitates a homeostasis between the reduced and oxidised glutathione disulphide (GSSG) forms. Optimal conditions in leaves have been described as approximately >90% GSH and <10% GSSG with any divergence from this ratio, namely an increase in GSSG relative to GSH, being indicative of oxidative stress (Foyer *et al.*, 2005). Biotic or abiotic stress can result in elevated production of ROS, such as O₂^{•-} or HO[•], that may oxidise GSH to GSSG. GSSG is also generated following detoxification of H₂O₂ into water – a reaction that uses GSH as a reducing agent and which can be catalysed by GSTs showing glutathione peroxidase activity (Dixon *et al.*, 1998). GSH also serves as a reductant in the synthesis of cysteine (Leustek, 2002; Smith *et al.*, 2010). Importantly, cellular homeostasis is maintained *via* the reduction of GSSG to GSH by glutathione reductase (GR; Figure 6.1) located in the cytosol, chloroplast, mitochondria and peroxisomes. Although there is very limited information available for cassava, a microarray analysis of cassava roots undergoing

PPD revealed an up-regulation of a predicted GST. Cassava GST (EST accession DT883580) was expressed 2-fold and 2.3-fold at 12 hr and 24 hr post-harvest, respectively, before returning to basal levels of expression. This appears to implicate a role of glutathione in cassava roots and possibly in the detoxification of H₂O₂, a known contributor to PPD (Reilly *et al.*, 2007). For clarity, throughout this thesis the term glutathione is used to denote both reduced (GSH) and oxidised (GSSG) forms; the appropriate abbreviations are used when specifically required.

6.1.4 Regulation of glutathione synthesis and involvement of γ -GCS

Induction and regulatory mechanisms for gene expression in glutathione synthesis is complex and remain to be fully mapped (Rausch *et al.*, 2007). *Arabidopsis* plants treated with jasmonic acid and heavy metals (e.g. copper and cadmium) responded by increasing transcription of *GSH1* and *GSH2* but not following exposure to H₂O₂ (Xiang & Oliver, 1998). However, increased H₂O₂ - brought about by treatment with aminotriazole (a catalase inhibitor) - did result in a 4-fold increase in glutathione (GSH) content in *Arabidopsis* suspension cultures (May & Leaver, 1993; May *et al.*, 1998) and a 3-fold increase in glutathione (specifically GSSG) in barley (*Hordeum vulgare* L.; Smith *et al.*, 1985). Interestingly, Queval *et al.* (2009) recently showed that H₂O₂-activated up-regulation of glutathione in catalase-deficient *Arabidopsis* (*cat2*) is associated with increased transcription of *ADENOSINE 5'-PHOSPHOSULPHATE REDUCTASE (APR)* and *SERINE ACETYLTRANSFERASE (SAT)* that are involved in cysteine synthesis. APR is located in plastids whereas SAT is present in various cellular locations, including the chloroplast, where it was strongly induced by H₂O₂. In *Arabidopsis* root cultures, application of L-cysteine in solution resulted in increased cysteine, γ -EC and GSH concentrations but decreased APR transcription and activity. Specifically, root cultures incubated with glutathione induced a significant decrease in APR mRNA levels and enzyme activity (Vauclare *et al.*, 2002). These data clearly interconnect the expression of genes involved in cysteine and glutathione synthesis, particularly the plastid located γ -GCS.

Regulation of glutathione synthesis also appears to involve post-translational modification of γ -GCS. Elucidation of the protein crystal structure from *B. juncea* (Hothorn *et al.*, 2006) revealed that, despite significant sequence differences between it and other γ -GCS, its conformation is surprisingly similar to that from *E. coli* (Hibi *et al.*, 2004). However, a plant-unique regulatory system was revealed that was based on two

disulphide bonds (CC1 and CC2). *In vivo* analysis of γ -GCS in *Arabidopsis* root extracts demonstrated that oxidative stress treatments, including H_2O_2 , altered the balance of oxidised (“active”) and reduced (“inactive”) forms (Hicks *et al.*, 2007). The oxidised enzyme is a homodimer but reduction of the CC2 bond results in dissociation into monomers and thus inactivation of the enzyme. CC2 is highly conserved in plants (and also in the evolutionary related α -proteobacteria) whereas CC1 is not, suggesting it is unlikely to be a second redox switch (Gromes *et al.*, 2008), although predictions suggest reduction of CC1 in *B. juncea* would allow a β -hairpin motif to shield the active site (Hothorn *et al.*, 2006). Whilst the CC2 redox switch seems a likely control for glutathione production, the compound that mediates the switch remains unknown. γ -GCS in extracts from *Nicotiana tabacum* cell suspension cultures were completely inhibited by glutathione (Hell & Bergmann, 1990) but reports postulate that glutathione is not directly involved due to its low reactivity and the involvement of thioredoxins or glutaredoxins (proteins that alter the structure and activity of target proteins; Meyer *et al.*, 2009) have been speculated (Gromes *et al.*, 2008; Rausch *et al.*, 2007). Clearly, the regulation and involvement of multiple redox, signalling and synthesis pathways is highly intricate and a comprehensive assessment and analysis is beyond the scope of this chapter. However, it is noteworthy that cysteine availability and synthesis as well as γ -GCS post-translational modifications have all been implicated in glutathione synthesis and regulation.

6.1.5 Over-expression of *GSH1* in planta

The important and multifarious roles of glutathione have led to the development of transgenic plants over-expressing *GSH1* to improve crop growth and sustainability to pathogen attack and other stresses. Over-expression of *GSH1* in *Arabidopsis* resulted in a 2-fold increase in GSH content and enhanced resistance to heavy metals (Xiang *et al.*, 2001). In Indian mustard, a 2-fold increase in glutathione improved plant tolerance to organic pollutants, such as the herbicide atrazine (Flocco *et al.*, 2004). Interestingly, it appears that whilst over-expression of *GSH1* results in enhanced glutathione content in plants, cellular targeting to either the cytosol or plastids impacts differently on cysteine flux. This was demonstrated in poplar hybrid (*Populus tremula* x *Populus alba*) transformed with bacterial γ -GCS targeted to the cytosol where a 10-fold increase in γ -EC and a 3-fold increase in glutathione occurred without affecting the GSH:GSSG ratio or depleting foliar cysteine pools (Noctor *et al.*, 1996). However, whilst a chloroplastic-targeted γ -GCS in poplar resulted in similar quantities of glutathione, increased amino

acid synthesis in the chloroplast was also detected (Noctor *et al.*, 1998). Although GSH is a feedback inhibitor of the cysteine pathway, the levels of mRNA and activities of enzymes involved in sulphate assimilation were only affected by exogenously applied GSH with a strong reduction in APR activity. These findings suggest sulphate assimilation in poplar was sufficient to accommodate increased γ -GCS expression and glutathione production (Hartmann *et al.*, 2004). Despite this, GSH content in γ -GCS transformed poplar leaf discs increased significantly when incubated with cysteine, indicating that cysteine supply remains, to some extent, a limiting factor for glutathione synthesis (Noctor *et al.*, 1996). Recent evidence obtained from measuring ^{35}S flux in transgenic poplars revealed that over-expressing γ -GCS directed to the cytosol, but not chloroplast-targeted, led to increased sulphur (i.e. as cysteine) into GSH (Scheerer *et al.*, 2009).

In contrast to other publications, Creissen *et al.* (1999) reported oxidative stress damage in transgenic tobacco expressing chloroplast-targeted γ -GCS. In-keeping with the aforementioned reports, a 3-fold increase in foliar glutathione content was measured but paradoxically foliar levels of H_2O_2 in transgenic lines were approximately double that in wild-type material. This manifested itself as leaf chlorosis or necrosis - a phenotype that was suggested to be caused by continuous oxidative damage as a consequence of failure of the redox-homeostasis in the chloroplast. Since these symptoms have not been observed in other plants, e.g. poplar, it was suggested that the growth habitats of different plants may reflect their varying capacity to oxidative stress (Creissen *et al.*, 2000). These detrimental effects of glutathione hyperaccumulation are important considerations for researchers seeking to improve plant defence *via* over-expression of *GSH1*. Interestingly, in *Streptococcus agalactiae*, a single bifunctional enzyme (γ -GCS-GS) is required for glutathione production and it is not inhibited by GSH (Janowiak & Griffith, 2005). This discovery poses an interesting prospect for increasing glutathione production *in planta*. In summary, the various publications cited here indicate that increased levels of glutathione can be achieved *via* over-expression of *GSH1 in planta* resulting in enhanced tolerance to various stresses. However, it is likely that glutathione accumulation will be limited by regulatory feedback mechanisms and/or cysteine availability. To what extent the absolute level of glutathione is dependent on the plant species has not been investigated (Maughan & Foyer, 2006).

6.2 RESEARCH OBJECTIVES

The goal is to utilise the modified *Agrobacterium*-mediated cassava transformation protocol (Chapter 4) to generate pDEST™-*AtGSH1* transgenic cassava with enhanced glutathione content. We hypothesise this will result in improved modulation of ROS and reduce oxidative damage. Generated material will be assessed to determine successful integration of the T-DNA, gene expression, PPD assays and samples will also be prepared to measure glutathione (GSH and GSSG), γ -EC and cysteine content. Note: *AtGSH1* used in these experiments was isolated from *Arabidopsis* (Table 3.1).

6.3 RESULTS

6.3.1 Identification of a putative cassava *GSH1* sequence

BLAST searches using *Arabidopsis GSH1* (TAIR accession AT4G23100) identified the homologous *GSH1* coding sequence in cassava nucleotide sequence databases (Section 2.4.10). The putative sequence, 1,572 nucleotides in length, has 74.4% sequence similarity to *Arabidopsis GSH1* but greater homology (89.4%) to the putative coding sequence from castor bean (*R. communis*), another species of Euphorbiaceae. Putative translated amino acid sequence of cassava *GSH1* has 80.6% and 89.9% similarity to *Arabidopsis* and castor bean, respectively. In both nucleotide and amino acid alignments most sequence variation arises at the 5'-end (Figure 6.2), which in *Arabidopsis GSH1* has been shown to encode the transit peptide (approximately 240 nucleotides in length) required to direct the gene product to plastids (Wachter *et al.*, 2005). This is the first report of an orthologous *GSH1* gene being identified in cassava.

6.3.2 Generation of pDEST™-AtGSH1 transgenic cassava

pDEST™-AtGSH1 transformed plantlets were produced from three independent batches of FEC cultivar TMS60444 (FEC6, FEC9 and FEC10) as described by Bull *et al.* (2009). 97 *in vitro* plantlets were screened using the rooting assay to ensure successful integration and expression of *hptII*, with only three plantlets failing to develop roots and thus presumed non-transgenic. The plantlets, catalogued into three groups (Group A → C) depending on the batch of FEC used and date transformed, were labelled with the group and then assigned a letter, A → Z and subsequently AA → AZ if more than 26 plants from a particular group were produced. For example, GCS:AK refers to the eleventh plant produced from Group A, whilst GCS:CAB is the 28th plant generated from Group C. 30 pDEST™-AtGSH1 transformed plantlets were selected for further analysis. Genomic DNA was extracted from *in vitro* leaf tissue (Section 2.3.1) and used in PCR-amplifications using primers specific to *hptII* (Hygro-For & Hygro-Rev; Table 2.1) and StPAT promoter/transgene (DESTSeqF1 & gshR2; Table 2.1; Section 2.1.2). The target *hptII* sequence was successfully amplified in all samples yielding a product of approximately 1 Kb (Figure 6.3). Similarly, the predicted sized product (approximately 1.7 Kb) using DESTSeqF1 & gshR2 primers was obtained from almost all samples with the exception of GCS:CI, GCS:CO and GCS:CAG (Figure 6.3). It is noteworthy that 31 plantlets transformed with the respective antisense construct (pDEST™-antiGSH1) also successfully passed the rooting test but due to time restrictions they were retained as *in vitro* stocks and not analysed further.

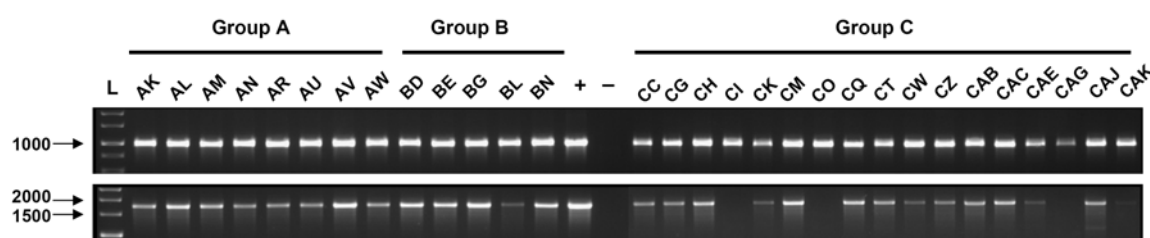


Figure 6.3 PCR-amplification products using pDEST™-AtGSH1 transformed *in vitro* plantlets. Products using *hptII* specific primers (Hygro-For & Hygro-Rev) (**upper image**) and StPAT promoter/transgene primers (DESTSeqF1 & gshR2) (**lower image**). Template plasmid DNA (+) and genomic DNA from wild-type TMS60444 (–) are shown. DNA ladder (bp; lane L) indicated. Plants grouped based on batch of FEC used and date transformed (Group A → C).

Genomic DNA isolated from the transgenic and wild-type *in vitro* plantlets was digested using *Hind*III (Section 2.3.1) and electrophoresed for Southern blot hybridisation with a DIG-labelled *hptII*-annealing probe (Sections 2.4.11-2.4.14). All samples, with the exception of GCS:AK, hybridised to the probe (Figure 6.4) indicating stable integration of the T-DNA in the plant genome. An unexpected 90% of samples possessed only one hybridised genomic fragment, whilst only 7% had two fragments and 3% (representing one line, GCS:BN) had four fragments in the plant genome. Approximately six independent lines could be identified amongst plants in Groups A and B (Figure 6.4a) and 10 lines were also identified from Group C (Figure 6.4b). Whether any lines identified in Groups A and B and Group C are the same was not investigated and only plantlets from the 10 lines identified in Group C (GCS:CC, GCS:CG, GCS:CH, GCS:CK, GCS:CM, GCS:CT, GCS:CZ, GCS:CAB, GCS:CAC and GCS:CAJ) were multiplied *in vitro* and grown to successfully establish plants in the glasshouse.

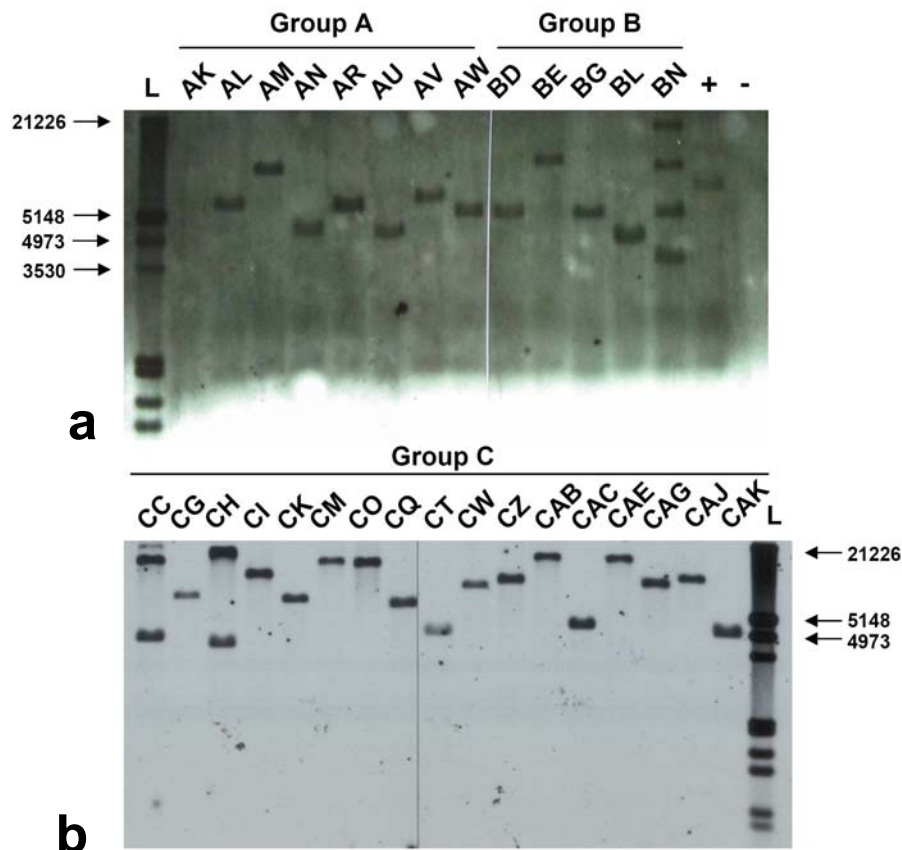


Figure 6.4 Southern blot hybridisation of *Hind*III digested genomic DNA from *in vitro* plantlets transformed with pDEST™-AtGSH1. Transformation Groups A and B (a) and transformation Group C (b). Samples hybridised to a *hptII*-annealing DIG-labelled probe. DNA ladder (bp; lanes L), plasmid DNA (+) and wild-type TMS60444 genomic DNA (–) shown.

6.3.3 Morphological characteristics of glasshouse cultivated plants

Plants representing the ten selected lines of pDEST™-AtGSH1 and also wild-type material were successfully cultivated in the glasshouse at the University of Bath. Mean root stock weight (Table 6.1; Figures 6.5 and 6.6) and plant height (Table 6.1; Figure 6.7) were not significantly different from wild-type plants, as determined by independent-samples *t*-tests (Table 6.1) and no phenotypic abnormalities were observed in any plants. Due to intermittent problems with the environment control systems some juvenile plants failed to develop and thus some lines (GCS:CK, GCS:CZ and GCS:CAJ) were represented by only one or two plants.

Table 6.1 Morphological data of glasshouse cultivated pDEST™-AtGSH1 transformed and wild-type plants.

Plant line	Number of plants	Mean plant height (cm), S.E. & (max. / min.)	<i>t</i> -test [†]	Number of roots	Mean root stock weight (g), S.E. & (max. / min.)	<i>t</i> -test [†]
GCS:CC	3	81.67, 0.67 (83 / 81)	p=0.317	8	55.67, 8.65 (68 / 39)	p=0.772
GCS:CG	4	65.75, 6.69 (80 / 50)	p=0.480	6	53.00, 5.57 (68 / 42)	p=0.940
GCS:CH	4	70.25, 3.15 (75 / 61)	p=0.683	9	61.25, 4.13 (73 / 55)	p=0.272
GCS:CK	2	67.00, 13.00 (80 / 54)	p=0.671	4	55.00, 1.00 (56 / 54)	p=0.768
GCS:CM	3	84.33, 2.19 (87 / 80)	p=0.221	7	56.33, 7.86 (67 / 41)	p=0.713
GCS:CT	4	73.50, 6.25 (85 / 56)	p=0.987	7	56.75, 1.84 (61 / 52)	p=0.565
GCS:CZ	1	69.00 (69)	§	1	55.00 (55)	§
GCS:CAB	4	72.50, 4.52 (84 / 69)	p=0.922	8	47.75, 1.75 (52 / 44)	p=0.551
GCS:CAC	4	78.25, 3.77 (83 / 67)	p=0.543	8	53.25, 4.57 (62 / 41)	p=0.909
GCS:CAJ	2	77.00, 7.00 (84 / 70)	p=0.755	4	64.50, 5.50 (70 / 59)	p=0.277
TMS60444	3	73.33, 7.27 (85 / 60)	n/a	3	52.33, 6.36 (65 / 45)	n/a

§ insufficient data for S.E. calculation or analysis

n/a not applicable (i.e. wild-type TMS60444)

† independent-samples *t*-test (transgenic line *versus* wild-type), significance $p \leq 0.05$ (*)

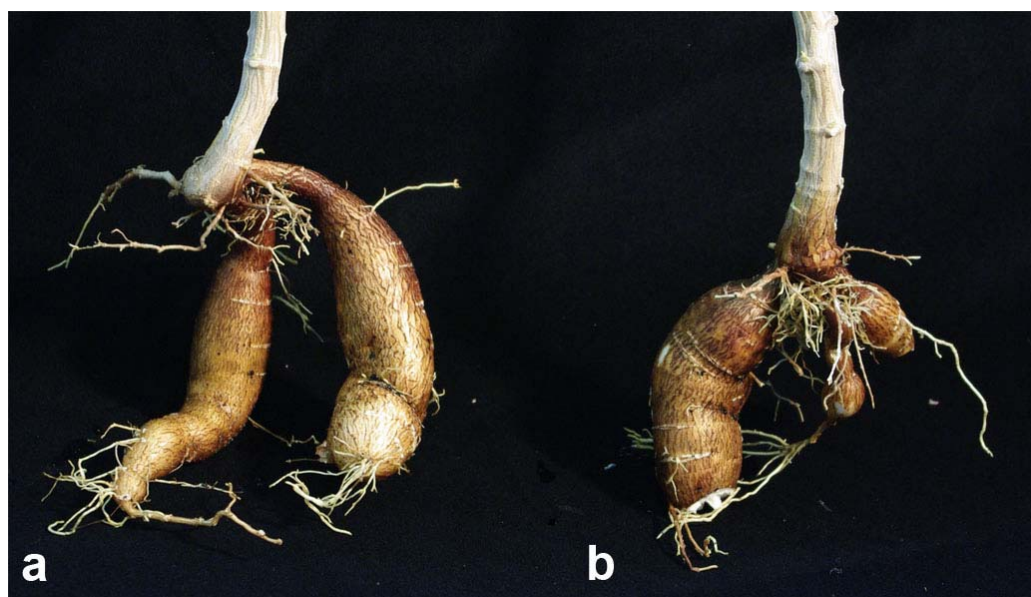


Figure 6.5 Example root stocks of pDEST™-AtGSH1 transformed (a) and wild-type cassava (b). No statistically significant difference was calculated between root stock weights of transgenic and wild-type material. Storage roots approximately 10 cm in length.

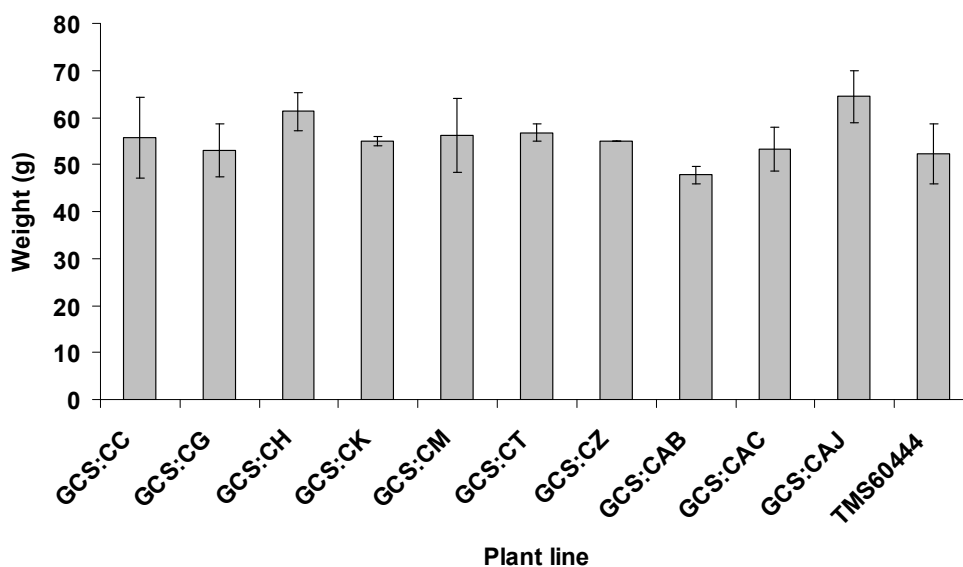


Figure 6.6 Mean root stock weight of glasshouse cultivated plants. Ten independent lines of pDEST™-AtGSH1 transformed plants and wild-type cassava (TMS60444). S.E. shown.

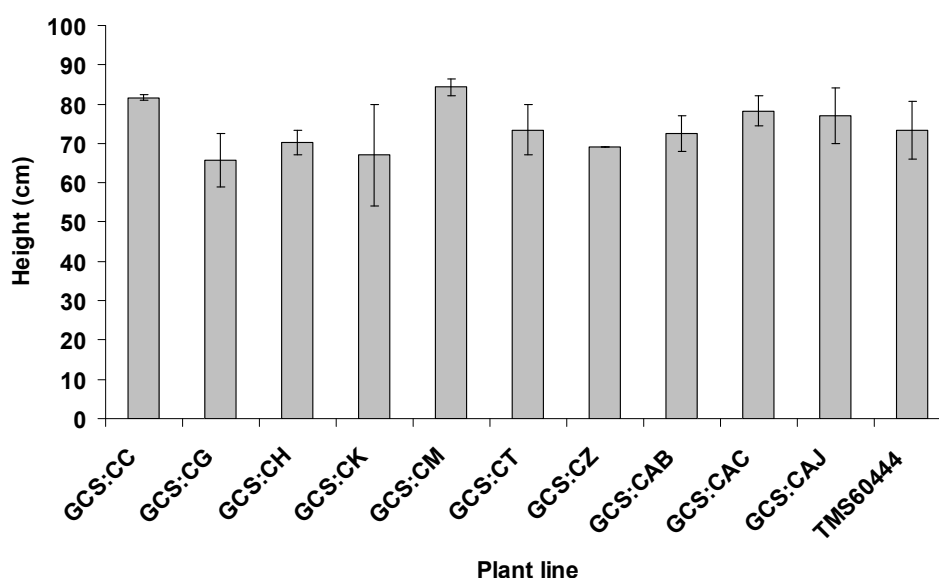


Figure 6.7 Mean height of glasshouse cultivated plants. Ten independent lines of pDEST™-*AtGSH1* transgenic plants and wild-type cassava (TMS60444) were assessed. S.E. shown.

6.3.4 Comparison of PPD symptoms between transgenic and wild-type roots

As discussed in Chapter 5, the assessment of PPD symptoms in the roots of glasshouse-cultivated plants (both transgenic and wild-type material) proved problematic due to the lack of symptom difference at the selected time points as well as considerable variation in symptoms. Similar observations were also apparent with the pDEST™-*AtGSH1* plants and are therefore not discussed in detail here. Six roots of wild-type TMS60444 plants were analysed at 0 hr, 24 hr and 96 hr time points (as described in Section 2.5.4) and revealed some discoloration (browning) of root samples at 24 hr post-harvest. All samples were severely deteriorated at 96 hr post-harvest (Figure 6.8). These general observations also apply to all the transgenic material, including line GCS:CC shown in Figure 6.9. Some of the root samples appear devoid of PPD symptoms but closer inspection revealed the tissue to be highly desiccated, chalky in texture, and thus the expected PPD process is unlikely to have occurred. This can be observed in, for example, TMS60444:BR2 (96 hr; Figure 6.8) and GCS:CC:AR1 (96 hr; Figure 6.9).

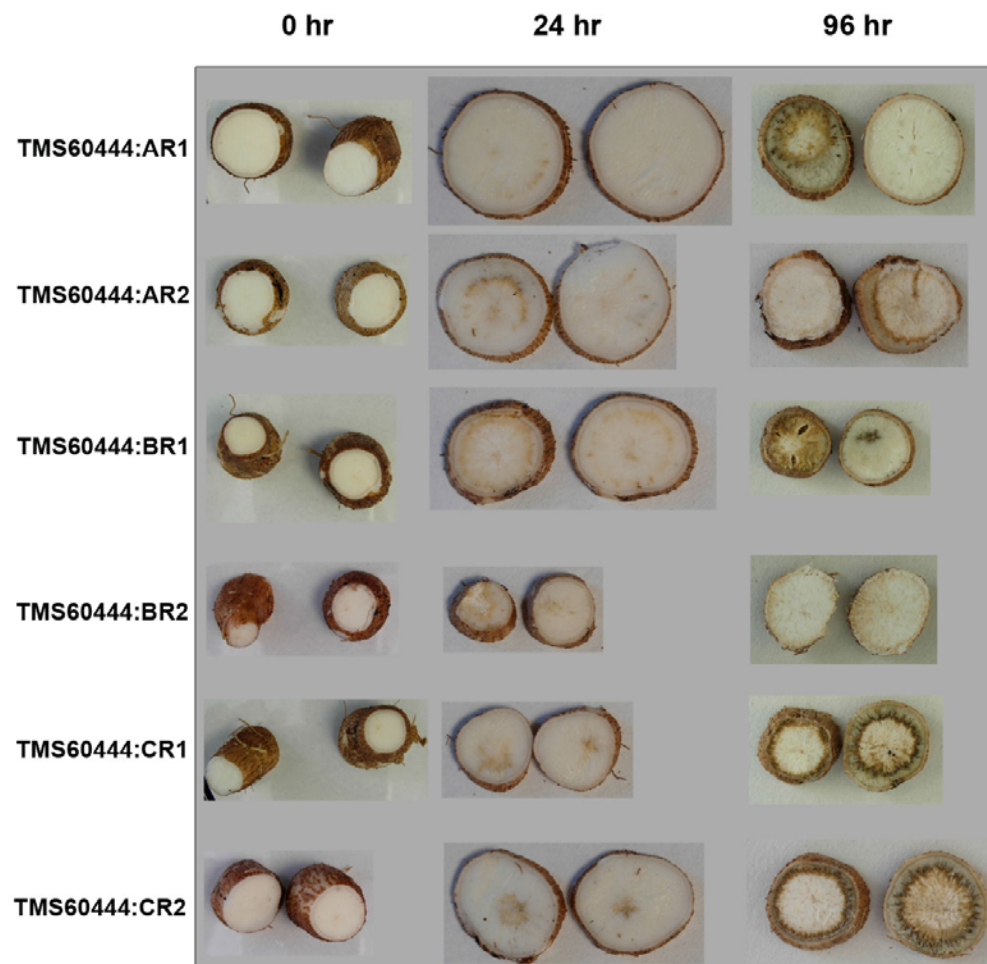


Figure 6.8 Wild-type TMS60444 root samples following harvest. Sections of roots shown 0 hr, 24 hr and 96 hr post-harvest. Six roots were harvested and assessed from three plants (TMS60444:A; TMS60444:B and TMS60444:C).

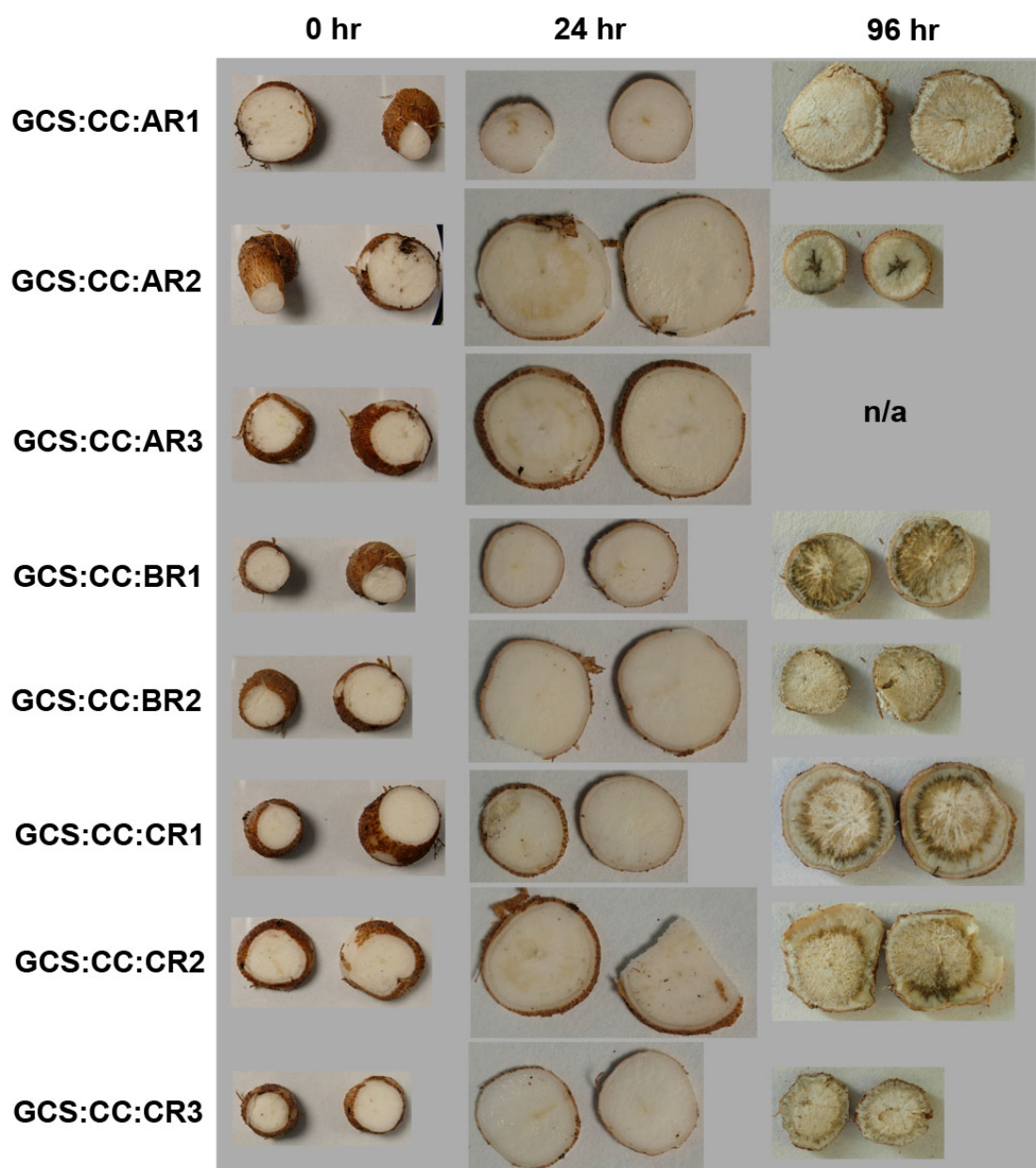


Figure 6.9 GCS:CC root samples following harvest. Sections of roots shown 0 hr, 24 hr and 96 hr post-harvest. Eight roots were harvested and assessed from three plants (GCS:CC:A; GCS:CC:B and GCS:CC:C). n/a: not available.

6.3.5 Comparative real-time PCR analysis of transgene expression

Total RNA was extracted from two storage roots (Section 2.6.1) of each line of pDEST™-AtGSH1 transformed mature plants, as well as from wild-type cassava. Unfortunately, in some cases the RNA was deemed too degraded for RT-PCR (Figure

6.10), probably a consequence of sub-optimal RNA extraction technique and/or degradation of the root tissue. Due to time constraints it was not possible to re-extract RNA from frozen tissue stocks and thus real-time PCR was performed on remaining samples. Ergo, lines GCS:CH, GCS:CK, GCS:CM, GCS:CT, GCS:CZ and GCS:CAB were represented by a single root each, whilst for lines GCS:CC, GCS:CAC and GCS:CAJ two roots were analysed. No real-time PCR data is currently available for line GCS:CG.

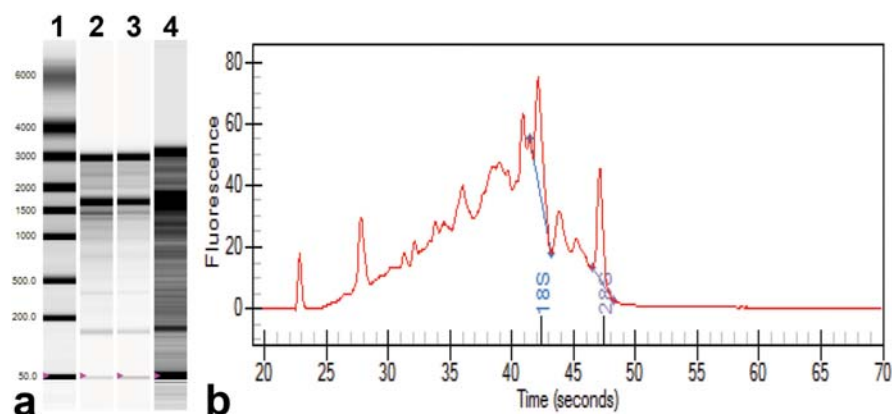


Figure 6.10 Analysis of RNA extracted from pDEST™-*AtGSH1* transgenic cassava roots. Selected samples - GCS:CK:BR1 (lane 2), GCS:CM:AR1 (lane 3), GCS:CT:AR1 (lane 4) and RNA ladder (bp; lane 1). 28S RNA (upper bands) and 18S RNA (lower bands). All samples 0 hr post-harvest **(a)**. Chromatogram of GCS:CT:AR1 highlighting the extent of RNA degradation **(b)**. Data obtained using the Experion™ Automated Electrophoresis System.

Standard *Taq*-based PCR (Section 2.1.2) successfully amplified the target sequence from GCS:CC:BR1 cDNA using amplification primers GCS Transgene F (located at position 1,470 – 1,490 in Figure 6.2) and Transgene R (Table 2.1; Figure 6.11). Minimal amplification from cDNA derived from wild-type TMS60444 root tissue was also observed although neither primer was predicted to anneal following BLAST comparisons and was thus likely to be the result of non-specific priming (Figure 6.11). The amplification product was not sequenced to verify these predictions. Whilst not ideal, real-time PCR using wild-type material recorded C_T values of approximately 40 and were thus regarded as background and not likely to influence the transgene specific amplification/data collection. Standard curve analysis revealed excellent reproducibility between technical replicates ($R^2 = 0.9968$) and an amplification efficiency of 76.7% ($E = 1.74$; Figure 6.12) that is highly similar to the *PP2A* reference gene (72.6%; Section 2.1.3).

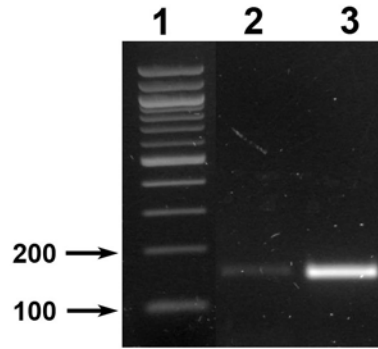


Figure 6.11 pDEST™-AtGSH1 PCR-amplification products. Amplification products using primers GCS Transgene F & Transgene R from cDNA derived from TMS60444 (lane 2) and (selected sample) GCS:CC:BR1 root tissue (lane 3). DNA ladder (bp) shown (lane 1). Root tissue sampled at 0 hr post-harvest.

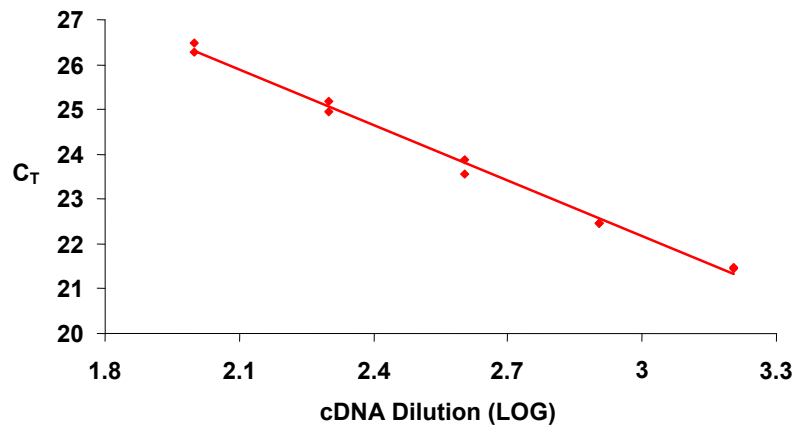


Figure 6.12 Standard curve analysis of real-time PCR amplification using transgene specific primers. Dilutions of GCS:CC:AR1 derived cDNA (selected sample) and transgene primers (GCS Transgene F & Transgene R). 0 hr: $y = -4.1258x + 34.552$, $R^2 = 0.9968$; 24 hr and 96 hr data not available.

Real-time PCR successfully detected transgene transcripts in all nine lines tested. Most significantly, a 10-fold up-regulation (compared to the reference gene) was observed in line GCS:CC, whilst an increase of 8.08 and 9.37-fold were also calculated in lines GCS:CM and GCS:CT, respectively. The lowest expression was observed in line GCS:CAB with only 0.57-fold increase. No transcripts were obtained from TMS60444 material ($2^{-\Delta CT} = 0.0003$), in-keeping with the expectation that amplification primers were

specific to the transgene/pDEST™-*AtGSH1* construct (Figure 6.13). As described in Section 2.6.4, two technical replicates were prepared for each biological sample and then two preparations were made for each replicate to ensure accurate C_T data were collected.

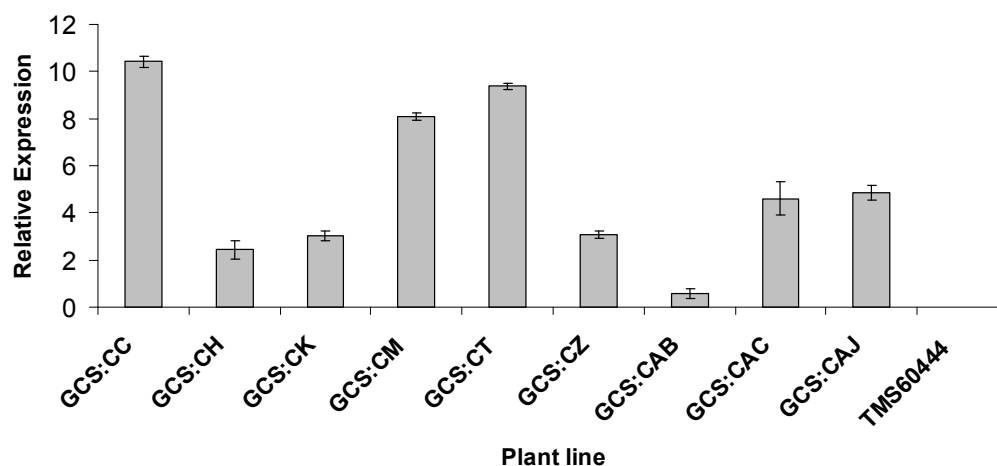


Figure 6.13 Mean relative expression of *AtGSH1* transgene comparative to *PP2A* in roots. Nine independent lines of pDEST™-*AtGSH1* transformed roots analysed 0 hr post-harvest. Wild-type data (TMS60444) and S.D. shown.

6.3.6 HPLC analysis of thiols in cassava roots and leaves

Samples from the primary storage root and leaves from each harvested pDEST™-*AtGSH1* transgenic plant were used to measure thiol content using HPLC (Sections 2.7.4 and 2.7.5). Quantities of cysteine, γ -EC, GSH and GSSG were accurately quantified in all samples at 0 hr post-harvest providing a comprehensive overview of glutathione production in cassava. In total, 32 roots and their respective leaf samples were analysed and compared to data obtained from six roots of three wild-type TMS60444 plants.

6.3.6.1 Cysteine content

The mean quantity of cysteine in transgenic roots was remarkably consistent across the different lines, ranging from 4.3-6.0 nmoles g^{-1} frozen weight (FzW) and was comparable to wild-type data (4.8 nmoles g^{-1} FzW; Table 6.2). In leaf material, however, greater variation and quantity in cysteine load was observed between the different lines (6.0-17.0 nmoles g^{-1} FzW), although these differences were not deemed statistically significant

(Table 6.2). Caution should be applied when drawing conclusions from these statistical analyses since it is possible that the relatively small sample size ($n=2-4$) is influencing data interpretation. The observed disparity in cysteine distribution between the roots and leaves occurred in both transgenic and wild-type plants where, on average, leaves had approximately twice as much cysteine compared to the corresponding roots (9.0 and 4.8 nmoles g^{-1} FzW, respectively; Table 6.2). This suggests that the transgene, whilst possibly affecting cysteine load, is not *per se* directly linked to variation in cysteine distribution in cassava leaf and root tissue. Interestingly, there is a medium positive correlation (Pearson Correlation) between the quantity of cysteine and glutathione in roots ($r=0.488$, $n=38$, $p=0.002$) and a large positive correlation in leaves ($r=0.766$, $n=34$, $p < 0.0005$). These correlations indicate that increased glutathione production *via* over-expression of the *AtGSH1* transgene is being accommodated by cysteine availability.

Table 6.2 Cysteine content in storage roots and leaves of pDEST™-*AtGSH1* transgenic and wild-type cassava.

Plant line	Mean Cys content	<i>t</i> -test [†]	Mean Cys content	<i>t</i> -test [†]
	(roots) nmoles g^{-1} FzW, S.E. (max. / min.)		(leaves) nmoles g^{-1} FzW, S.E. (max. / min.)	
GCS:CC	4.3, 0.67 (5 / 3)	$p=0.598$ $n=3$	11.3, 0.67 (12 / 10)	$p=0.155$ $n=3$
GCS:CG	4.3, 0.48 (5 / 3)	$p=0.474$ $n=4$	6.0, 1.00 (9 / 5)	$p=0.107$ $n=4$
GCS:CH	5.8, 0.85 (8 / 4)	$p=0.366$ $n=4$	9.8, 1.03 (12 / 7)	$p=0.650$ $n=4$
GCS:CK	6.0, 1.00 (7 / 5)	$p=0.329$ $n=2$	8.5, 0.50 (9 / 8)	$p=0.766$ $n=2$
GCS:CM	5.3, 0.88 (7 / 4)	$p=0.626$ $n=3$	11.0, 0.00 (11 / 11)	$p=0.158$ $n=3$
GCS:CT	5.0, 0.71 (7 / 4)	$p=0.854$ $n=4$	11.3, 1.44 (13 / 7)	$p=0.302$ $n=4$
GCS:CZ	5.5, 1.50 (7 / 4)	$p=0.604$ $n=2$	12.0 §	§ $n=1$
GCS:CAB	5.0, 0.58 (6 / 4)	$p=0.844$ $n=4$	8.5, 1.04 (11 / 6)	$p=0.762$ $n=4$
GCS:CAC	6.0, 0.91 (8 / 4)	$p=0.273$ $n=4$	11.0, 0.71 (13 / 10)	$p=0.178$ $n=4$
GCS:CAJ	4.5, 1.50 (6 / 3)	$p=0.793$ $n=2$	17.0, 1.00 (18 / 16)	$p=0.017^*$ $n=2$
TMS60444	4.8, 0.54 (6 / 3)	n/a $n=6$	9.0, 1.56 (11 / 7)	n/a $n=3$

§ insufficient data for S.E. calculation or analysis

n/a not applicable (i.e. wild-type TMS60444)

† independent-samples *t*-test (transgenic line *versus* wild-type), significance $p \leq 0.05$ (*)

6.3.6.2 γ -EC, GSH and GSSG content

The mean quantity of γ -EC in lines of transgenic root ranged between 7.0 nmoles g^{-1} FzW in line GCS:CZ to 13.0 nmoles g^{-1} FzW in line GCS:CC. Generally the quantity of γ -EC in transgenic roots was greater than in wild-type material, although this was statistically significant only in lines GCS:CG, GCS:CM and GCS:CT (Table 6.3). Similarly, the corresponding leaf tissue of the aforementioned lines also represented the least and greatest quantity of γ -EC detected amongst the ten transgenic lines, although GCS:CK also had only 7.0 nmoles g^{-1} FzW (Table 6.3). As for the root analysis, the levels of γ -EC were consistently greater than the mean content in wild-type leaves (5.0 nmoles g^{-1} FzW; Table 6.3). Quantities were statistically greater in all lines, with the exception of GCS:CG and GCS:CK. These data clearly indicate an increased production of γ -EC in leaves of transgenic plants compared to wild-type plants - a conclusion that corroborates the real-time PCR data (Section 6.3.5) and confirming successful over-expression of the *AtGSH1* transgene. Not surprisingly, there was a strong positive correlation between the quantity of γ -EC and glutathione in both roots ($r=0.761$, $n=38$, $p < 0.0005$) and leaves ($r=0.553$, $n=34$, $p=0.001$), indicating that increased production of γ -EC (the intermediate product in glutathione synthesis) is being utilised by GSH-S and thus enhancing levels of glutathione.

Table 6.3 γ -EC content in storage roots and leaves of pDEST™-AtGSH1 transgenic and wild-type cassava.

Plant line	Mean γ -EC content	<i>t</i> -test [†]	Mean γ -EC content	<i>t</i> -test [†]
	(roots) nmoles g ⁻¹ FzW, S.E. & (max. / min.)		(leaves) nmoles g ⁻¹ FzW, S.E. & (max. / min.)	
GCS:CC	13.0, 2.08 (17 / 10)	p=0.104 n=3	16.7, 1.45 (19 / 14)	p=0.003 ** n=3
GCS:CG	10.5, 1.04 (13 / 8)	p=0.014 * n=4	8.0, 1.47 (12 / 5)	p=0.181 n=4
GCS:CH	9.5, 1.19 (12 / 7)	p=0.147 n=4	14.5, 1.04 (17 / 12)	p=0.001 *** n=4
GCS:CK	9.5, 1.50 (11 / 8)	p=0.357 n=2	7.0, 0.00 (7 / 7)	p=0.219 n=2
GCS:CM	11.0, 1.73 (14 / 8)	p=0.031 * n=3	17.3, 3.93 (25 / 12)	p=0.038 * n=3
GCS:CT	9.5, 0.96 (12 / 8)	p=0.045 * n=4	8.3, 0.48 (9 / 7)	p=0.024 * n=4
GCS:CZ	7.0, 2.00 (9 / 5)	p=0.937 n=2	7.0 §	n=1
GCS:CAB	7.3, 0.25 (8 / 7)	p=0.920 n=4	13.0, 0.71 (14 / 11)	p=0.001 *** n=4
GCS:CAC	11.5, 1.56 (15 / 8)	p=0.066 n=4	11.3, 1.32 (14 / 9)	p=0.017 * n=4
GCS:CAJ	8.0, 2.00 (10 / 6)	p=0.758 n=2	14.0, 1.00 (15 / 13)	p=0.009 ** n=2
TMS60444	7.2, 0.37 (8 / 6)	n/a n=5	5.0, 1.00 (7 / 4)	n/a n=2

§ insufficient data for S.E. calculation or analysis

n/a not applicable (i.e. wild-type TMS60444)

† independent-samples *t*-test (transgenic line *versus* wild-type), significance $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***)

On average, 93.7% of glutathione in transgenic roots is GSH and comparable to 88.7% in wild-type tissue (Figure 6.14). Interestingly, the GSH:GSSG ratio in leaves is markedly lower with only 65.3% of glutathione in transgenic material being GSH, which was similarly observed in wild-type leaves (66.5% GSH; Figure 6.15). The altered ratio in cassava leaves raises interesting questions regarding the redox state of glutathione in roots and leaves of cassava and whether foliar tissue is reflecting an abiotic and/or biotic stress (please see discussion).

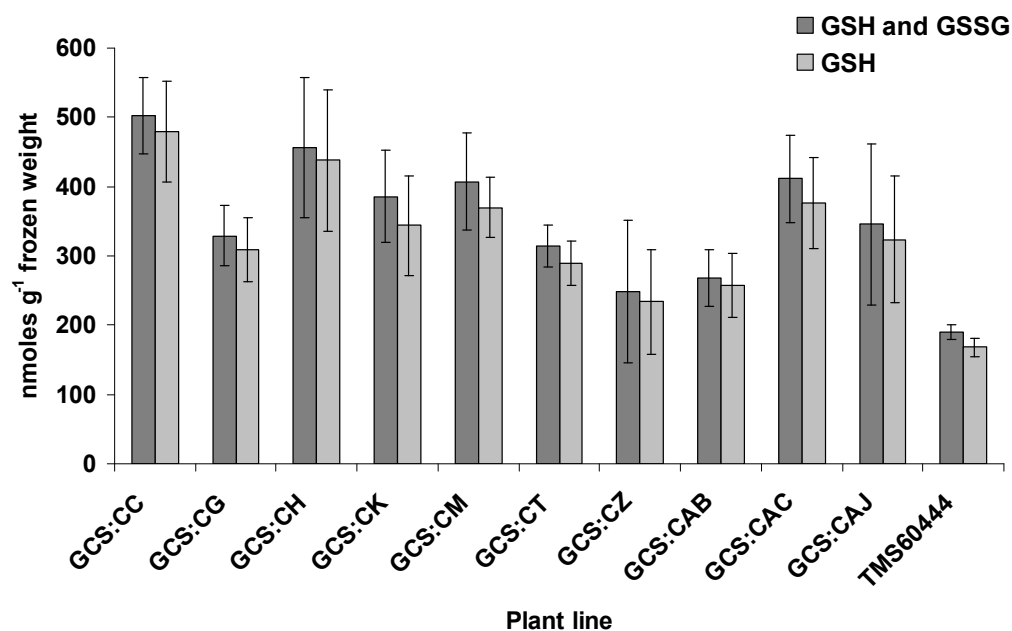


Figure 6.14 Glutathione in cassava storage roots. Mean total glutathione (GSH and GSSG) and GSH in storage roots representing ten pDESTTM-AtGSH1 transgenic lines. Wild-type (TMS60444) data and S.E. shown.

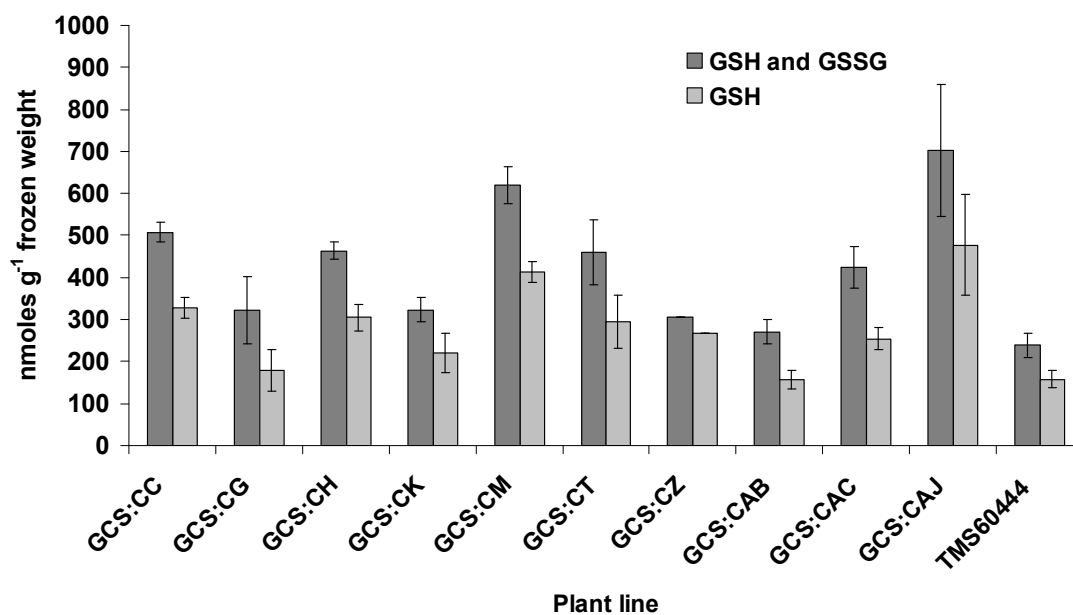


Figure 6.15 Glutathione in cassava leaves. Mean total glutathione (GSH and GSSG) and GSH in leaves representing ten pDESTTM-AtGSH1 transgenic lines. Wild-type (TMS60444) data and S.E. shown.

In all transgenic plant lines there was an increase in glutathione content in both roots and leaves. This increase was statistically significant in lines GCS:CC, GCS:CG, GCS:CT and GCS:CAC, as determined by independent-samples *t*-test (Table 6.4; Figure 6.16). The greatest increase was measured in line GCS:CC that had 2.65-fold more glutathione than wild-type plants. In leaves, a 2.95 fold increase was observed in line GCS:CAJ but this was not deemed statistically significant (Table 6.4; Figure 6.16). It is likely that biological variation i.e. differing quantities of glutathione in roots of the same line, coupled with the relatively small sample size, resulted in some lines appearing on average to have large quantities of glutathione but which were not statistically significant, such as GCS:CAJ. Analysis of additional biological samples should provide more comprehensive information regarding the independent transgenic lines. Only plant lines GCS:CC and GCS:CAC had statistically significant increased levels of glutathione in both storage roots and leaves (Table 6.4; Figure 6.16).

Table 6.4 Glutathione in storage roots and leaves of pDEST™-AtGSH1 transgenic cassava compared to wild-type plants.

Plant Line	Fold increase (Roots)	<i>t</i> -test [†]	Fold increase (Leaves)	<i>t</i> -test [†]
GCS:CC	2.65	p=0.025 * n=3	2.13	p=0.002 ** n=3
GCS:CG	1.74	p=0.006 ** n=4	1.36	p=0.427 n=4
GCS:CH	2.41	p=0.078 n=4	1.94	p=0.001 *** n=4
GCS:CK	2.14	p=0.202 n=2	1.36	p=0.144 n=2
GCS:CM	1.66	p=0.087 n=3	2.60	p=0.002 ** n=3
GCS:CT	1.66	p=0.002 ** n=4	1.93	p=0.066 n=4
GCS:CZ	1.31	p=0.671 n=2	1.29	§ n=1
GCS:CAB	1.41	p=0.057 n=4	1.14	p=0.476 n=4
GCS:CAC	2.17	p=0.036 * N=4	1.78	p=0.031 * n=4
GCS:CAJ	1.82	p=0.407 n=2	2.95	p=0.197 n=2

§ insufficient data for analysis

† independent-samples *t*-test (transgenic line *versus* wild-type), significance $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***)

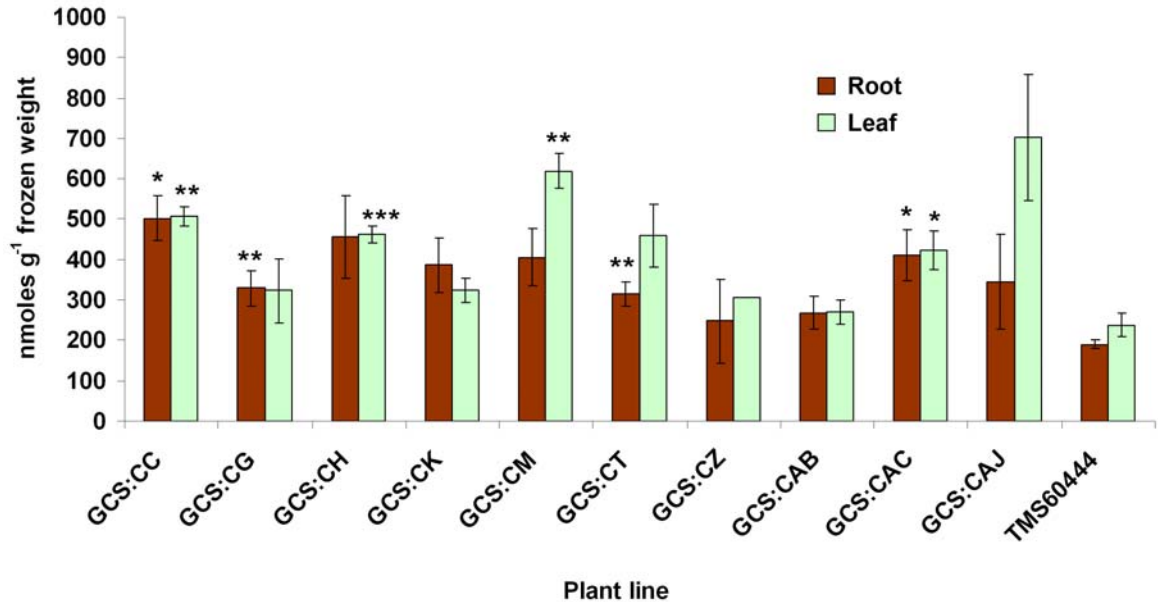


Figure 6.16 Summary of glutathione content of pDEST™-AtGSH1 transformed and wild-type (TMS60444) cassava. Roots and leaves of ten independent plant lines and wild-type (TMS60444) cassava. Statistical significance (independent-samples *t*-test, transgenic line *versus* wild-type) indicated: $p \leq 0.05$ (*), $p \leq 0.01$ (**) and $p \leq 0.001$ (***) and S.E. shown.

6.4 DISCUSSION

This chapter describes the successful creation and characterisation of cassava over-expressing *AtGSH1* following *Agrobacterium*-mediated transformation of cultivar TMS60444 with pDEST™-*AtGSH1*. This is the first report of cassava transformed with genes involved in glutathione synthesis and unique in also measuring GSH, GSSG, γ -EC and cysteine content of storage roots and leaves. The results raise interesting topics of discussion regarding glutathione production in cassava as well as the function(s) of glutathione in prolonging the shelf-life of storage roots and PPD.

6.4.1 Increased glutathione content does not appear to affect symptoms of PPD.

Visual assessments of roots sampled at 0 hr, 24 hr and 96 hr post-harvest revealed no apparent variation in PPD symptoms between transgenic and wild-type material. However this finding is somewhat inconclusive since the selected time points were possibly inappropriate for assessing deterioration in glasshouse-cultivated storage roots; the onset and rate of PPD is likely to be far more rapid than in larger, field-grown roots. The reader is directed to the discussion in Chapter 5 where similar observations and

constraints to the analysis of pDEST™-MecAPX2 transformed plants were appraised and that are also relevant to the pDEST™-AtGSH1 transgenic cassava presented here. Notwithstanding the set-backs in sampling, it appears that increased glutathione content *per se* does not noticeably minimise root deterioration at 24 hr or 96 hr post-harvest. However, there are possible exceptions that certainly merit further investigation. For example, roots of GCS:CC - the most highly expressing line - analysed 24 hr after harvest do appear to be less symptomatic compared to wild-type roots, but clearly a more robust system for assessment is required to draw definitive conclusions.

It should be remembered that H₂O₂ flux was not measured in cassava samples and thus it is conceivable that modulation of H₂O₂ accumulation may have been improved but not to an extent that resulted in preventing deterioration at the selected time points. Measuring H₂O₂ is all the more important when one considers that Creissen *et al.* (1999) observed necrosis and enhanced H₂O₂ production in tobacco plants over-expressing plastid targeted γ -GCS. Herschbach *et al.* (2010) also reported similar oxidative damage in transgenic poplars but only in plants over 4½ months in age. However, these symptoms were not observed in cassava and therefore, even if levels of H₂O₂ were raised, available evidence suggests that it was not detrimental to plant growth. An enhancement of H₂O₂ modulation may also be closely related to increased expression of scavenging enzymes such as APX, CAT and/or GSTs (Dixon & Edwards, 2010). Reilly *et al.* (2007) reported a 2.5 fold increase in transcription of a predicted GST 12-24 hr post-harvest, possibly implicating GSH in harvested roots/PPD. This gradual expansion of knowledge raises exciting possibilities to investigate and substantiate the proposed link between glutathione production and PPD in cassava, with particular focus on gathering gene expression data for enzymes such as GST and measurements of H₂O₂.

6.4.2 Is there a restriction on glutathione accumulation in pDEST™-AtGSH1 cassava?

Real-time PCR amplification of the transgene in roots of line GCS:CC revealed a 10-fold increase compared with the reference gene (*PP2A*) and yet there was only a 2.65-fold increase in glutathione content; a 1.9-fold mean increase was calculated amongst all transgenic lines. A 2-fold increase in glutathione was also obtained in *Arabidopsis* (Xiang *et al.*, 2001), *B. juncea* (Flocco *et al.*, 2004) and a 3-fold increase in poplar (Noctor *et al.*, 1996), in-keeping with the data presented here. Collectively, these data suggest that glutathione production is limited by regulatory feedback pathways such as post-

translation modifications of γ -GCS and involvement in cysteine production is highly controlled (Noctor *et al.*, 2011) and that transgenic cassava is no exception. Interestingly, Liedschulte *et al.* (2010) recently showed a 20-fold increase in glutathione production in tobacco using the bifunctional enzyme γ -GCS-GSH-S isolated from *S. thermophilus* and without having any apparent deleterious impact on plant growth. This enzyme is neither redox regulated nor sensitive to feedback inhibition by glutathione, allowing unprecedented levels of glutathione to accumulate. The bifunctional enzyme originally isolated from *S. agalatae* (Janowiak & Griffith, 2005) was requested for incorporation into this project but due to time constraints and problems experienced with the transformation protocol (Chapter 4) further exploration with the clone was prevented. The use of bifunctional enzymes present an interesting prospect for future experiments, albeit for academic research rather than for commercialisation of cassava.

6.4.3 Why is the GSH:GSSG redox in roots and leaves different?

The optimal GSH:GSSG balance in plants has been reported to be >90% GSH and <10% GSSG (Noctor *et al.*, 2011). This is commensurate with the data collected from cassava roots but there is a disparity with the ratio measured in leaves where a greater proportion of GSSG was detected. One simple explanation may be that the baseline ratio of glutathione in cassava leaves is more imbalanced than in other plants. However, it seems more probable that the unusual GSH:GSSG ratio is the consequence of oxidative stress in foliar tissue (Mahmood *et al.*, 2010). Since wild-type plants have the same foliar glutathione ratio as transgenic plants it seems implausible that the transgene is responsible for modification of the redox balance. It also seems unlikely that the different ratio is a consequence of stress *via* harvesting itself since the leaf tissue and roots were sampled simultaneously to prevent any signal transduction (Ghanta *et al.*, 2011; Şahin & Tullio, 2010) and thus minimise the possibility of different accumulation patterns. Paradoxically, leaf tissue was immersed in liquid nitrogen within seconds of harvest, whereas root tissue was peeled, grated and then frozen – a process that may take a couple of minutes and thus far more likely to incur oxidative stress and damage than the leaves. If increased foliar GSSG content is the consequence of oxidative damage, further experiments need to be conducted to understand what the cause of the stress may be. For example, light, nutrient depletion and drought may all trigger an oxidative stress response (Ball *et al.*, 2004; Noctor *et al.*, 2011).

6.4.4 Is thiol distribution and accumulation in leaves and roots offering insights into transport and signalling?

The strong positive correlation between cysteine availability and glutathione content in leaves could indicate that foliar glutathione synthesis is driving production and regulation throughout the plant. The weaker positive correlation in roots, despite the root-specific *StPAT* promoter, is commensurate with this hypothesis. It is known that in plants the uptake of sulphates from the soil is directed to the leaves *via* the xylem for cysteine production (Smith *et al.*, 2010; Takahashi *et al.*, 2011) and is therefore the likely focal point for enhanced glutathione content in cassava. Sulphate assimilation is therefore possibly constrained in the roots, which may be reflected in the minimal variation in cysteine content. Poplar leaf discs over-expressing *GSH1* increased glutathione production when incubated in a cysteine rich solution (Noctor *et al.*, 1996), highlighting the impact of cysteine availability on GSH synthesis. It should be noted that no transcript data is available for transgene expression in cassava leaves and it is predicted (based on evidence in Chapter 5) that the transgene is being over-expressed in foliar tissue. Additionally, the cellular localisation of γ -GCS in roots has not been determined but is presumed to be in amyloplasts, where the cellular dynamics and redox state have not been elucidated. The presumed transport in the phloem of glutathione to roots coupled with enhanced transcription of transgene in roots may be leading to increased gene regulation and sulphur flux in roots (Li *et al.*, 2006). In poplar this has been linked to higher rates of phloem loading and possibly increased defence gene expression as well as a decrease in transcription of *APR* (Herschbach *et al.*, 2010), a finding that is coherent with the knowledge that GSH is involved in cysteine synthesis regulation (Vauclare *et al.*, 2002).

6.4.5 Summary and future work

The findings presented here provide an in-sight into glutathione synthesis in cassava and serve to direct future research. The primary objective is to prolong the shelf-life of cassava storage roots but by undertaking additional experiments a broader characterisation of glutathione synthesis in cassava should be achieved. The results presented in this chapter utilised material harvested at 0 hr and therefore it is necessary to investigate transgene expression (i.e. real-time PCR) and glutathione content (i.e. HPLC analysis) in the root and leaf tissue sampled at 24 hr and 96 hr post-harvest, as well as to measure γ -GCS activity in key lines (G. Creissen, *pers. comm.*). Incorporating

a greater number of biological samples should also provide sufficient data for comprehensive statistical analysis.

Since there appears to be some discrepancy in transgene expression patterns, namely the possibility that it is not root specific and/or being influenced by the neighbouring *CaMV35S* promoter (see Chapter 5 for further discussion), it would seem prudent to investigate gene expression profiles in more detail and in particular extend real-time PCR assessments to incorporate endogenous genes. Following identification of key lines there is also scope to expose the plants to various stresses, including H_2O_2 , and also perhaps biotic stresses such as cassava mosaic viruses since enhanced glutathione content may have significant benefits to plant defence other than the intended desire to modulate H_2O_2 accumulation and PPD. Future work may also address in more detail the genes involved in the cysteine assimilation pathway and sulphur flux between leaves and roots since it has been shown that glutathione influences cysteine synthesis and may cast light on the interaction between roots and leaves. Ultimately, these approaches should broaden our knowledge of glutathione production in cassava, allowing improved knowledge as well as opportunities to extend the shelf-life of cassava.

7 IMPROVING ROS-MODULATION AND TRANSGENE EXPRESSION IN CASSAVA

7.1 INTRODUCTION

The enzymes and antioxidants involved in ROS modulation in plants provide various means to regulate oxidative stress. In addition to *GSH1* required for glutathione biosynthesis and *APX* involved in H_2O_2 breakdown (please see Chapters 5 and 6 for further information) other genes of interest include *GALACTURONIC ACID REDUCTASE* and *SUPEROXIDE DISMUTASE*.

7.1.1 Galacturonic acid reductase in ascorbate production

Ascorbate is a multifaceted molecule with roles in stress response, serving as a reducing agent in H_2O_2 degradation catalysed by *APX* (Smirnoff, 2000; Chapter 5). Several biosynthetic pathways to ascorbate have been described that comprise various intermediate reactions and products. The Smirnoff-Wheeler pathway, which uses D-glucose as the primary substrate, has been studied using *Arabidopsis* mutants and appears to be the predominant route for ascorbate production in plants. The “galacturonate pathway”, however, utilises D-galacturonic acid - a component released from cell wall pectins during hydrolysis - as a substrate that is reduced to L-galacturonic acid by galacturonic acid reductase (*GalUR*; EC 1.1.1.19). This is converted to ascorbate via L-galactono- γ -lactone (Smirnoff *et al.*, 2001; Valpuesta & Botella, 2004). Improving ascorbate content in plants for plant stress resistance and human nutrition has led to concerted efforts to over-express genes in the pathways, including *GalUR* (Ishikawa *et al.*, 2006). Over-expression of *GalUR* isolated from strawberry (*F. ananassa*) and under control of the *CaMV35S* promoter resulted in a 2-3 fold increase in ascorbate in *Arabidopsis* (Agius *et al.*, 2003). In potato tubers (*S. tuberosum* L. cultivar Taedong Valley) a 1.6-2 fold increase in ascorbate content was detected by HPLC and the plants displayed enhanced resistance to abiotic stress including MV treatment (Hemavathi *et al.*, 2009). Interestingly, over-expression of strawberry *GalUR* assessed in isolated hairy roots of tomato (*S. lycopersicum* cultivar Money Maker) and treated with D-galacturonic acid produced a 2.5 fold increase in ascorbate content, suggesting the substrate may be a limiting factor in its biosynthesis (Oller *et al.*, 2009). A microarray study of harvested cassava roots revealed up-regulation (2.4-fold at 24 hr) of an enzyme involved in ascorbate biosynthesis (UDP-glucose dehydrogenase) but which is not involved in the

galacturonate pathway (Reilly *et al.*, 2007). *GalUR* expression has not been identified in cassava but based on the aforementioned publications it seems reasonable to hypothesise that an increase in ascorbate can be achieved *via* over-expression of *GalUR* and that may improve tolerance to ROS.

7.1.2 Superoxide dismutase

Superoxide dismutase (SOD; EC 1.15.1.1) is the first line of defence against ROS catalysing the dismutation of $O_2^{\bullet -}$ into oxygen and H_2O_2 ($2 O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$). $O_2^{\bullet -}$ arises in cellular compartments where an electron transport chain is present, especially the chloroplast, mitochondria and peroxisomes. Phospholipid membranes are impermeable to $O_2^{\bullet -}$ and therefore it is important SOD exists in various locations. SOD enzymes are classified into three groups according to their metal cofactor and which have differing cellular sites i.e. copper/zinc SOD (Cu/ZnSOD) are present in chloroplasts and the cytosol, manganese SOD (MnSOD) in mitochondria and peroxisomes, whereas iron SOD (FeSOD) are in chloroplasts (Alscher *et al.*, 2002). In cassava, two full-length *Cu/ZnSOD* cDNA have been isolated. *MecSOD1* (NCBI accession AF170297) was cloned and characterised from cell cultures and is highly expressed in intact stems and storage roots. There are only low levels of expression in leaves and petioles and no expression in fibrous roots. Expression of cytosolic *MecSOD1* was up-regulated following stress treatments such as MV and wounding (Lee *et al.*, 1999). Cytosolic *MecSOD2* (NCBI accession AY642137) was also isolated from cassava suspension cultures and transcript analysis revealed high expression in stems but low levels in leaves and roots. Similarly to *MecSOD1*, the gene was highly induced by abiotic stresses including MV exposure, high temperature (37°C) and wounding in leaf tissue (Shin *et al.*, 2005). These remain the only characterised *SOD* genes in cassava, although mitochondrial MnSOD activity has been detected in harvested cassava roots (Isamah *et al.*, 2003). Interestingly, total SOD activity appears not to increase significantly following harvest, although an increase in Cu/ZnSOD activity was detected within 24 hr post-harvest before returning to basal levels (Isamah *et al.*, 2003; Reilly *et al.*, 2004). These findings are supported by microarray analysis that failed to detect significant increases (≥ 1.8 fold) in gene transcripts in harvested cassava root (Reilly *et al.*, 2007).

The important role of SOD in combating ROS accumulation has resulted in the development of plants with improved oxidative defence. Over-expression of a chloroplast-targeted *Cu/ZnSOD* from pea resulted in tobacco plants with improved

photosynthetic capacity at high light intensity and reduced cellular damage following MV treatment (Gupta *et al.*, 1993a). Interestingly, although not surprisingly, the increase in SOD activity and thus H₂O₂ production was compensated by enhanced expression of endogenous *APX* (Gupta *et al.*, 1993b). Over-expression of a cytosolic *Cu/ZnSOD* from the mangrove plant *Avicennia marina* in rice led to improved tolerance to MV-induced oxidative stress and drought stress (Prashanth *et al.*, 2008). These selected studies highlight the capacity of transgenic plants to tolerate oxidative stress and by boosting *MecSOD2* expression in cassava roots may assist in minimising oxidative damage and PPD.

7.1.3 *MecPX3* encodes a secretory peroxidase in cassava

Among the many genes expressed during plant defence responses are class III POX (EC 1.11.1.7). They belong to a multigene family and have diverse roles, participating in lignin and suberin formation and metabolism of ROS (Almagro *et al.*, 2009). The haem-containing enzymes are located in vacuoles and cell walls and are able to oxidise phenolic substrates (e.g. hydroxycoumarins) at the expense of H₂O₂, thus aiding the modulation of ROS to minimise cellular damage (Passardi *et al.*, 2007). The biotic or abiotic stress induced expression of POX is conferred by various *cis* regulatory elements in the promoter sequence (Sasaki *et al.*, 2007) and in *Arabidopsis* the majority of class III POX are expressed in roots (Tognolli *et al.*, 2002; Welinder *et al.*, 2002). Interestingly, *MecPX3* (NCBI accession AY973612) - identified during a microarray study of harvested cassava roots - is reported to encode a secretory peroxidase whose expression is up-regulated and peaks (based on selected time points) at 24 hr after sampling (Reilly *et al.*, 2007; Figure 7.1). Whilst there is only limited information regarding *MecPX3*, the spatial and temporal expression of the gene suggested a link between root specific and wound induced expression (Figure 7.1). Given the lack of characterised cassava root and PPD specific promoters, the regulatory region of *MecPX3* was identified to have enormous potential to drive transgene expression in future experiments.



Figure 7.1 Expression profile of *MecPX3* in cassava. Northern blot of *MecPX3* mRNA (**upper image**) in root tissue assessed 0 hr, 12 hr, 24 hr and 72 hr post-harvest. Leaf tissue – wounded but attached leaves (lane A), wounded and detached leaves (lane D) and control, unwounded attached leaves (lane CK). 18S RNA loading control (**lower image**). Figure modified from Reilly *et al.* (2007).

7.2 RESEARCH OBJECTIVES

The goal is to utilise the modified *Agrobacterium*-mediated cassava transformation protocol (Chapter 4) to generate pDEST™-*GalUR*, pDEST™-anti*GalUR*, pDEST™-*MecSOD2* and pDEST™-anti*SOD* transgenic cassava. The over-expression constructs should result in improved modulation of ROS accumulation and thus may reduce oxidative damage. The plants will be screened to identify independent transgenic lines and preliminary assessment for PPD will be undertaken. In addition, plantlets transformed with pDEST™-*GUSPlus* will be generated to allow studies into the *StPAT* promoter in cassava. Lastly, the promoter region of *MecPX3* will be isolated from cassava with the prospect to study expression profiles.

7.3 RESULTS

7.3.1 Generation of pDEST™-*GalUR* transgenic cassava

Two independent batches of FEC cultivar TMS60444 (FEC6 and FEC9) were transformed as described by Bull *et al.* (2009) using pDEST™-*GalUR*. 59 *in vitro* plantlets were generated of which 57 successfully passed the rooting test, representing 97% of tested plants. Transgenic plantlets were labelled as outlined in previous chapters. It is noteworthy that eight plantlets transformed with the antisense construct (pDEST™-anti*GalUR*) also successfully passed the rooting test but were not analysed further due to time constraints. Genomic DNA was extracted from *in vitro* leaf tissue (Section 2.3.1) of 30 selected pDEST™-*GalUR* plantlets and used in PCR-amplifications using primers to *hptII* (Hygro-For & Hygro-Rev; Table 2.1) and *StPAT* promoter/transgene (DESTSeqF1 & garR1, Table 2.1; Section 2.1.2). The target *hptII* and transgene sequence were successfully amplified in all samples, generating fragments of the correct

size (approximately 1 Kb and 1.2 Kb, respectively). As predicted, neither product was generated from wild-type TMS60444 cassava DNA samples (Figure 7.2).

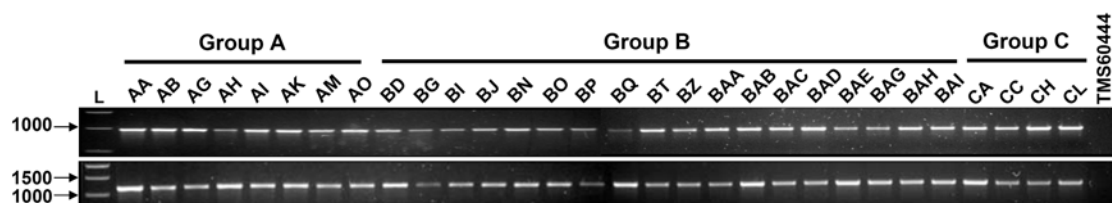


Figure 7.2 PCR-amplification products from pDEST™-GalUR transformed *in vitro* plantlets. Products using *hptII* specific primers (Hygro-For & Hygro-Rev) (**upper image**) and *StPAT* promoter/transgene primers (DESTSeqF1 & garR1) (**lower image**). Products from genomic DNA of wild-type TMS60444 (negative control) are shown. DNA ladder (bp) indicated (lane L). Plants grouped based on batch of FEC used and date transformed (Group A → C).

Genomic DNA isolated from 19 selected transgenic and wild-type *in vitro* plantlets was digested using *HindIII* and electrophoresed for Southern blot hybridisation with a DIG-labelled *hptII*-annealing probe (Sections 2.4.11-2.4.14). All transgenic samples hybridised to the probe (Figure 7.3) indicating stable integration of the T-DNA in the plant genome. 74% (equal to 14 samples) revealed hybridisation to only one genomic fragment, plant samples GalUR:AI, GalUR:BD and GalUR:CC each had two hybridised fragments and only a single sample (GalUR:AO) had three. Nine independent lines were identified (GalUR:AM, GalUR:AO, GalUR:BP, GalUR:BAA, GalUR:BAC, GalUR:BAD, GalUR:BAG, GalUR:CH and GalUR:CL) and plantlets multiplied *in vitro* (approximately five plantlets per line) for transfer to soil and growth in the glasshouse (Section 2.5.3).

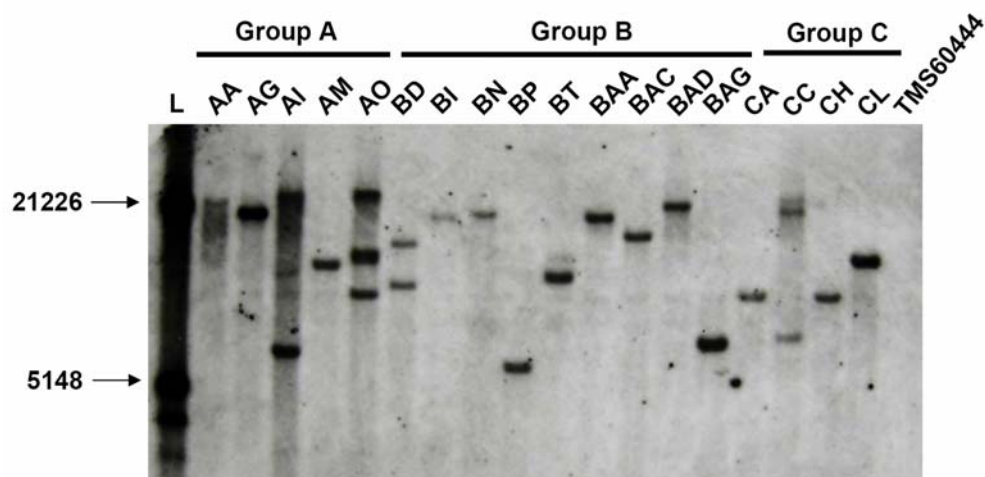


Figure 7.3 Southern blot hybridisation of *Hind*III digested genomic DNA from *in vitro* plantlets transformed with pDEST™-*GalUR*. All samples hybridised to a *hptII*-annealing DIG-labelled probe and plants grouped based on batch of FEC used and date transformed (Group A → C). DNA ladder (bp; lane L) and wild-type TMS60444 genomic DNA (negative control) shown.

7.3.2 Morphology of pDEST™-*GalUR* transgenic plants

25 pDEST™-*GalUR* transgenic and five wild-type TMS60444 plants were successfully grown in the glasshouse (Table 7.1). Storage roots were harvested from all plants with the exception of *GalUR*:AM:B and *GalUR*:BP:D where the roots were too small for analysis. Additionally, plantlets from line *GalUR*:CL became irretrievably contaminated during the early stages of propagation in soil and therefore were discarded. All harvested plants had a normal phenotype but, as occurred in similar experiments, failings with the systems that regulate the environmental conditions resulted in some losses and thus different numbers of biological replicates were available for each line. Most affected were *GalUR*:AM, *GalUR*:AO and *GalUR*:CH that are represented by two plants each. Generally there was no statistically significant difference in the mean plant height of transgenic lines compared with wild-type plants, with the exception of *GalUR*:BAA and *GalUR*:BAG (Table 7.1; Figure 7.4). However, based on available evidence it seems unlikely this has any biological implication and probably due to ongoing modifications to the growth regime. There was no statistically significant difference in the mean root stock weight of transgenic line and wild-type material (Table 7.1; Figure 7.5).

Table 7.1 Morphological characteristics of glasshouse cultivated pDEST™-GalUR plants.

Plant line	Number of plants	Mean plant height (cm), S.E. & (max. / min.)	t-test [†]	Number of roots	Mean root stock weight (g), S.E. & (max. / min.)	t-test [†]
GalUR:AM	2	48.3, 2.19 (51 / 44)	p=0.963	3	41.5, 4.50 (46 / 37)	p=0.272
GalUR:AO	2	53.0, 3.00 (56 / 50)	p=0.193	4	47.0, 3.00 (50 / 44)	p=0.492
GalUR:BP	5	54.8, 3.48 (64 / 45)	p=0.125	7	58.3, 6.65 (71 / 42)	p=0.737
GalUR:BAA	5	53.6, 0.68 (56 / 52)	p=0.017 *	7	50.8, 4.32 (66 / 42)	p=0.600
GalUR:BAC	3	50.7, 3.84 (55 / 43)	p=0.516	5	47.7, 6.94 (60 / 36)	p=0.487
GalUR:BAD	3	48.3, 3.48 (54 / 42)	p=0.970	4	40.0, 10.79 (60 / 23)	p=0.243
GalUR:BAG	4	55.8, 1.11 (58 / 53)	p=0.009 **	5	40.5, 5.55 (56 / 32)	p=0.140
GalUR:CH	2	51.5, 3.50 (55 / 48)	p=0.367	3	38.0, 7.00 (45 / 31)	p=0.193
TMS60444*	5	48.2, 1.66 (52 / 42)	n/a	7	55.0, 6.38 (71 / 32)	n/a

[†] independent-samples *t*-test (transgenic line *versus* wild-type), significance $p \leq 0.05$ (*) and $p \leq 0.01$ (**)

n/a not applicable (i.e. wild-type TMS60444)

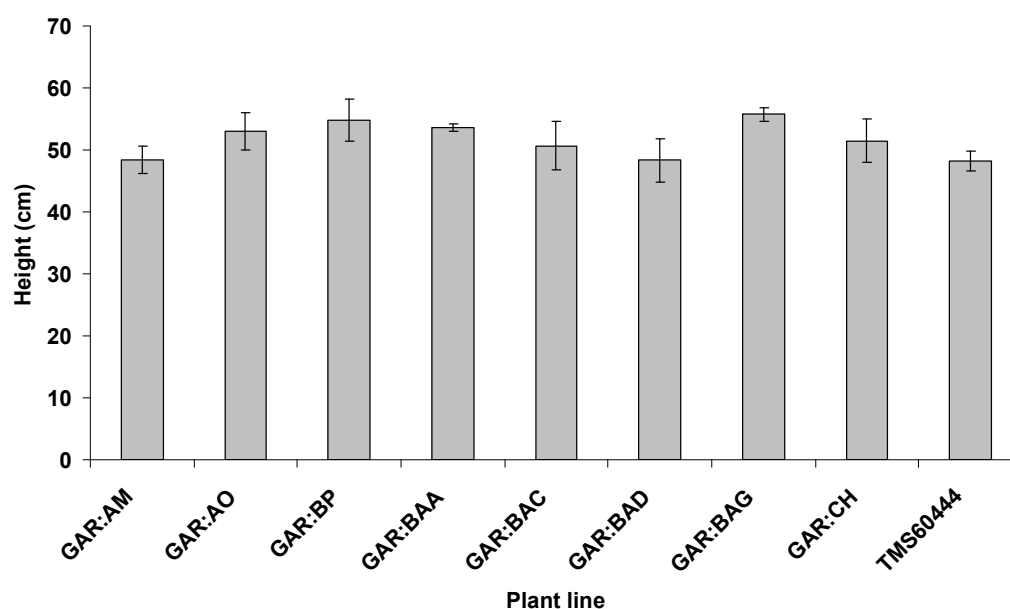


Figure 7.4 Mean height of glasshouse cultivated pDEST™-*GalUR* plants. Eight independent lines of pDEST™-*GalUR* transgenic plants and wild-type cassava (TMS60444) were assessed. S.E. shown.

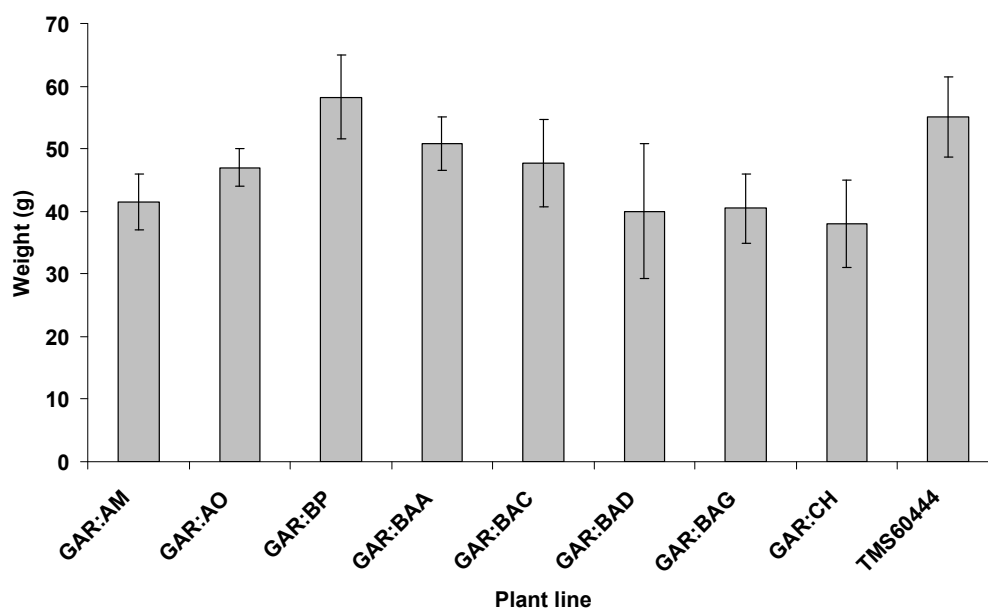


Figure 7.5 Mean root stock weight of glasshouse cultivated pDEST™-*GalUR* plants. Eight independent lines of pDEST™-*GalUR* transformed plants and wild-type cassava (TMS60444). S.E. shown.

7.3.3 PPD assay of pDEST™-GalUR transgenic plants

The 38 storage roots from glasshouse-cultivated transgenic plants and seven roots of wild-type TMS60444 were assessed for symptoms of PPD at 0 hr, 24 hr and 72 hr post-harvest. It is noteworthy that unlike in previous experiments (Chapters 5 and 6), roots were surveyed at 72 hr rather than 96 hr, to minimise complications regarding the rate of PPD. As expected, no PPD symptoms were observed in either transgenic or wild-type roots at the 0 hr time point (Figures 7.6 and 7.7). Similarly, almost all root samples observed 24 hr post-harvest had no symptoms with mild discoloration in a few sections of both transgenic and wild-type root, for example GalUR:BP:BR1 and TMS60444:ER1, respectively. At 72 hr post-harvest most tissue had symptoms indicative of PPD and less general deterioration/decay compared to previous observations at 96 hr. Furthermore, the gradation in symptom severity between adjacent slices seems more apparent. For example, in GalUR:BP:BR2 the section closest to the wounded end (left in photograph) has more severe symptoms than the more distal section (right in photograph; Figure 7.7). Whilst this is only an observation and does not apply to all root sections, it does support the supposition that at 72 hr post-harvest PPD is a more active process with roots less likely to undergo decay and desiccation - events common place in samples harvested at 96 hr.

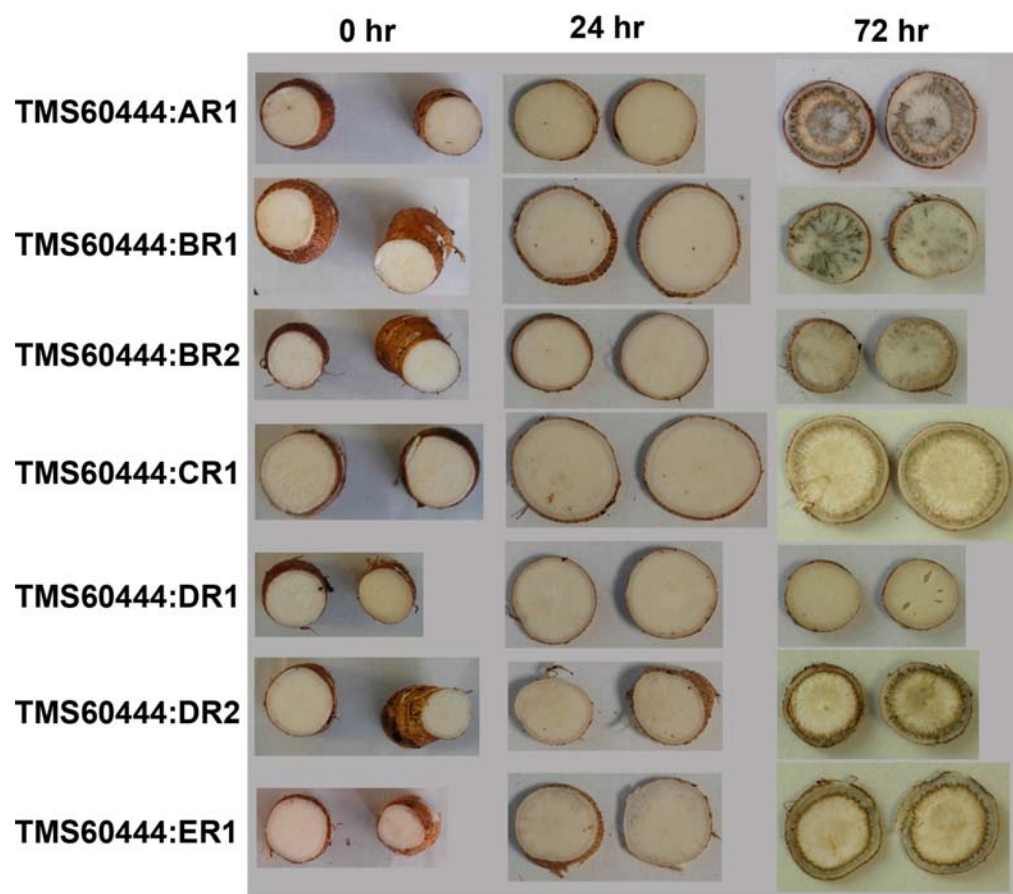


Figure 7.6 Wild-type TMS60444 roots following harvest. Sections of roots shown 0 hr, 24 hr and 72 hr post-harvest. Seven roots were harvested and assessed from five plants (TMS60444:A; TMS60444:B; TMS60444:C; TMS60444:D and TMS60444:E).

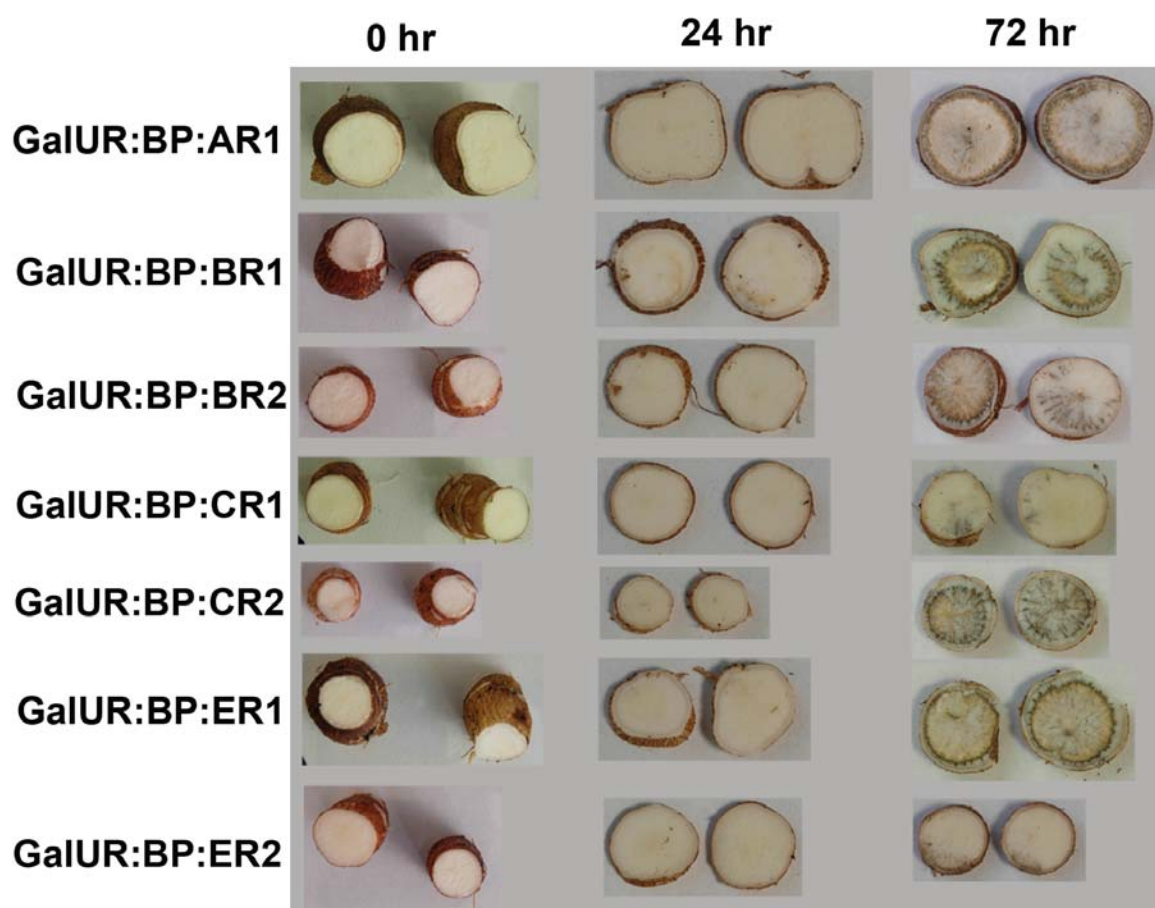


Figure 7.7 Transgenic GalUR:BP roots following harvest. Sections of roots shown 0 hr, 24 hr and 72 hr post-harvest. Seven roots were harvested and assessed from four plants (GalUR:BP:A, GalUR:BP:B, GalUR:BP:C and GalUR:BP:E).

7.3.4 Generation of pDEST™-MecSOD2 and pDEST™-GUSPlus transgenic cassava

32 *in vitro* plantlets transformed with pDEST™-MecSOD2 and 26 plantlets containing pDEST™-GUSPlus were confirmed transgenic following a rooting assay. These results were substantiated by the production of the expected sized fragment following PCR-amplification with primers specific to *hygII* (Hygro-F & Hygro-R; Table 2.1) in all pDEST™-MecSOD2 samples (Figure 7.8) but failed in GUSPlus:AG, GUSPlus:AP and GUSPlus:AS (Figure 7.9). PCR of these samples need to be repeated to understand whether this was due to an experimental error or incomplete integration of the T-DNA. Failure to generate a product using primers specific to the *StPAT* promoter (Pat-Pst F & Pat-Pml R; Table 2.1) was also observed in GUSPlus:AL and GUSPlus:AT, although the other 23 tested samples yielded expected sized fragments. Southern blot hybridisation

using a *hptII*-annealing DIG-labelled probe was performed and as predicted hybridised to all representative samples of pDEST™-*MecSOD2* samples. However, due to poor separation of the DNA during electrophoresis (data not shown) it was not possible to determine independent lines, although 13/15 samples appeared to hybridise to only a single genomic fragment. Southern blot hybridisation of selected pDEST™-*GUSPlus* plantlets confirmed integration of *hptII* in the plant genome in 18/20 samples. For samples GUSPlus:AI and GUSPlus:AJ the DNA appears to be degraded and thus hybridisation to the probe did not occur (Figure 7.10). 18 *in vitro* plantlets were also produced following transformation of FEC (FEC9) with pDEST™-antiSOD. Curiously, 56% of the tested samples were not transgenic, which is an uncharacteristically poor success rate and further investigation is required to determine whether this is due to sub-optimal experimental conditions or possibly a transgene effect.

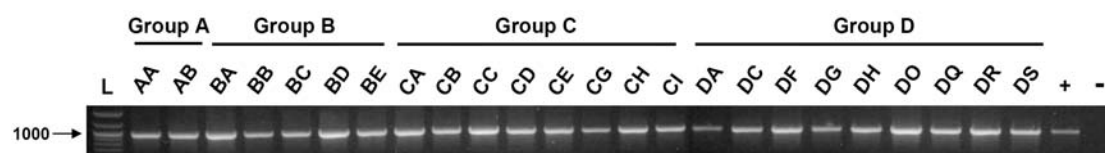


Figure 7.8 PCR-amplification products from pDEST™-*MecSOD2* transformed *in vitro* plantlets. Products using *hptII* specific primers (Hygro-For & Hygro-Rev). Amplification from genomic DNA from wild-type TMS60444 (negative control; lane —) and plasmid DNA template (positive control; lane +) are shown. DNA ladder (bp; lane L). Plants grouped based on batch of FEC used and date transformed (Group A → D).

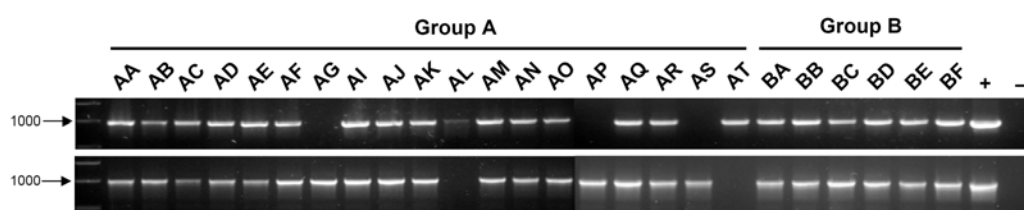


Figure 7.9 PCR-amplification products from pDEST™-*GUSPlus* transformed *in vitro* plantlets. Products using *hptII* specific primers (Hygro-For & Hygro-Rev) (**upper image**) and StPAT promoter primers (Pat-Pst F & Pat-Pml R) (**lower image**). Products from genomic DNA from wild-type TMS60444 (negative control; lane -) and plasmid DNA template (positive control; lane +) are shown. DNA ladder (bp) indicated. Plants grouped based on batch of FEC used and date transformed (Group A and B).

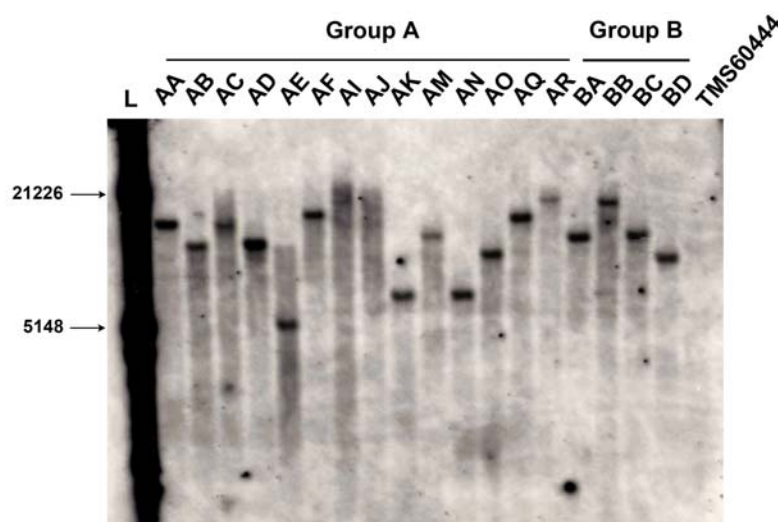


Figure 7.10 Southern blot hybridisation of *Hind*III digested genomic DNA from *in vitro* plantlets transformed with pDESTTM-*GUS*Plus. All samples hybridised to a *hptII*-annealing DIG-labelled probe and plants grouped based on batch of FEC used and date transformed (Group A and B). DNA ladder (bp; lane L) and wild-type TMS60444 genomic DNA (negative control) shown.

7.3.5 Isolation and characterisation of the *MecPX3* promoter

Since discovering that *MecPX3* gene expression occurs only in root tissue and increased following harvest, there has been interest to isolate the promoter region. Preparatory work was undertaken by K. Reilly (University of Bath) who created and screened a lambda-based genomic library of MCOL22 (Sections 2.3.2-2.3.4). A number of plaques were identified that purportedly contained the *MecPX3* gene and from which DNA was extracted (Section 2.3.4) for further analysis.

7.3.5.1 Lambda-cloned genomic DNA isolation

DNA was extracted from plaques (named PX3ii and PX3iii; Section 2.3.4) of lambda-cloned genomic DNA and aliquots were successfully digested with *Bam*HI, *Hind*III, *Kpn*I and *Pst*I restriction enzymes (Section 2.4.4). The products were resolved on a TAE agarose gel (Section 2.4.2; Figure 7.11) and prepared for Southern blot hybridisation using a [α -³²P]-dCTP labelled probe of *MecPX3* cDNA (Section 2.4.12). The probe hybridised successfully to fragments of PX3ii DNA, confirming presence of the *MecPX3* gene (Figure 7.11). The probe failed to hybridise to the PX3iii samples, suggesting either a technical error or a problem with the initial screening phase. Attempts were undertaken

to sequence the PX3ii genomic clone (courtesy of Lark Sequencing, UK) but for unknown reasons this technique proved ineffective and therefore alternative strategies were considered to isolate the promoter.

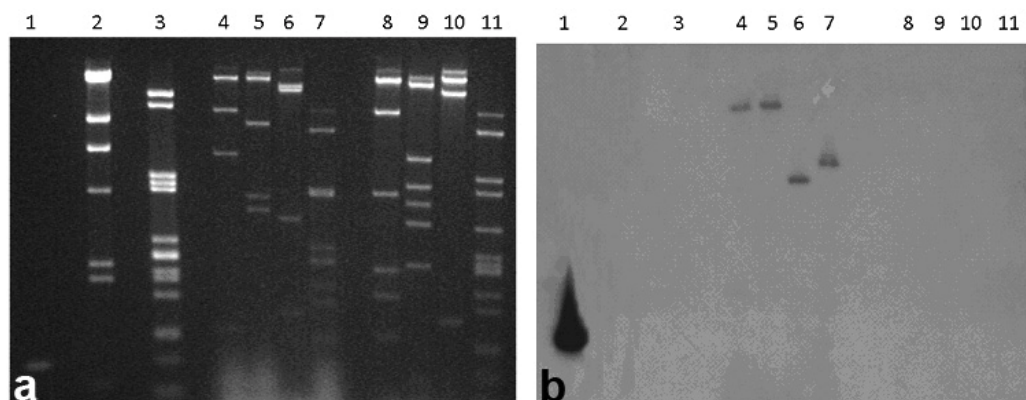


Figure 7.11 Restriction fragment length polymorphism of lambda DNA of cassava genomic library (a) and Southern blot (b). *MecPX3* cDNA (lane 1), *HindIII* and *PstI* digested lambda DNA (lanes 2 & 3, respectively). *BamHI*, *HindIII*, *KpnI* and *PstI* digested DNA (lanes 4-7) PX3ii and (lanes 8-11) PX3iii, respectively. Hybridization using a [α - 32 P]-dCTP labelled probe of *MecPX3* cDNA.

7.3.6 GenomeWalker™ isolation of *MecPX3* promoter

Following unsuccessful attempts to derive the *MecPX3* promoter from lambda libraries the GenomeWalker™ kit using cultivar BRA337 was adopted (Section 2.3.5). Although the coding sequence of *MecPX3* is known (NCBI accession AY973612) it was necessary to first characterise the transcript sequence to ensure primers designed for use with the GenomeWalker™ kit were optimally positioned i.e. not spanning exon-exon junctions. Sequence specific amplification primers for the coding region of *MecPX3* were generated (PX3-GSP3 & PX3-GSP9; Table 2.1) with the reverse primer positioned approximately 90 bp upstream of the putative termination codon to avoid an AT-rich region at the 3'-end of the coding sequence. PCR-amplification products (approximately 1.5 Kb) were successfully generated (Sections 2.1.2 and 2.4.1), cloned (Sections 2.2.1 and 2.2.6) and sequenced (Section 2.4.9), allowing identification of three introns in the isolated sequence (Figure 7.12). Interestingly, five nucleotide discrepancies were also identified throughout the sequence but whether these were technical errors or possibly due to differences between cultivars (published sequence used CM2177-2 whereas the sequence presented here was from TMS60444) was not addressed.

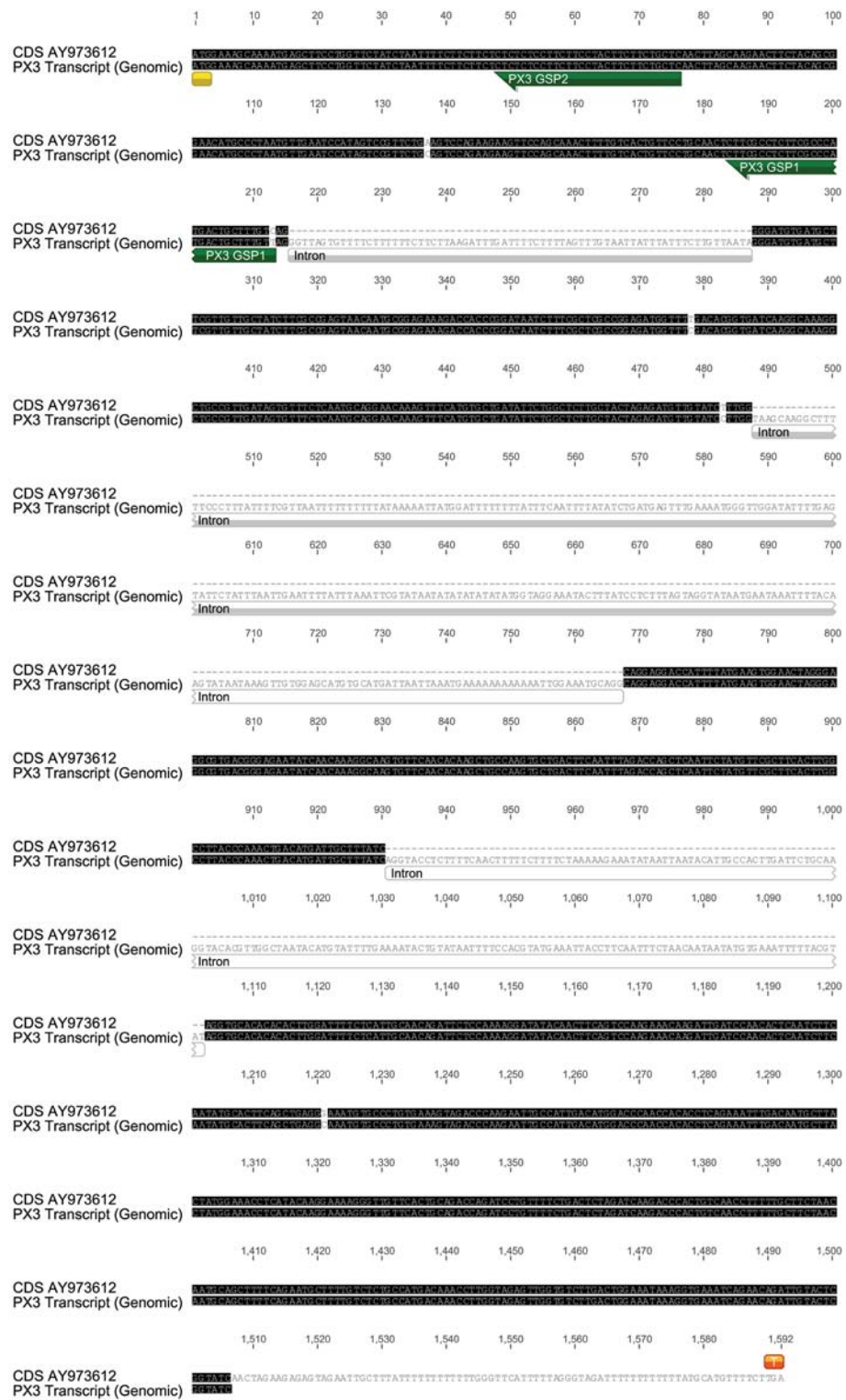


Figure 7.12 Nucleotide alignment of *MecPX3* putative coding sequence from cassava. Genomic DNA sequence from TMS60444 and coding sequence from CM2177-2 (NCBI accession AY973612). Introns (grey rectangles), predicted termination codon (orange box), start codon (yellow box) and amplification primers (green arrows) shown.

There is 65.6% sequence identity between cassava coding sequence (AY973612) and the most homologous gene in *Arabidopsis* (AT2G18980) and 78.9% to that in castor bean (NCBI accession XM_002532757.1). Expression maps of AT2G18980 revealed gene transcription primarily in root tissue and up-regulation to various stimuli including wounding and oxidative stress (Figure 7.13). These data suggest that the *MecPX3* promoter could be a prospective candidate to regulate transgene expression in investigations of cassava PPD.

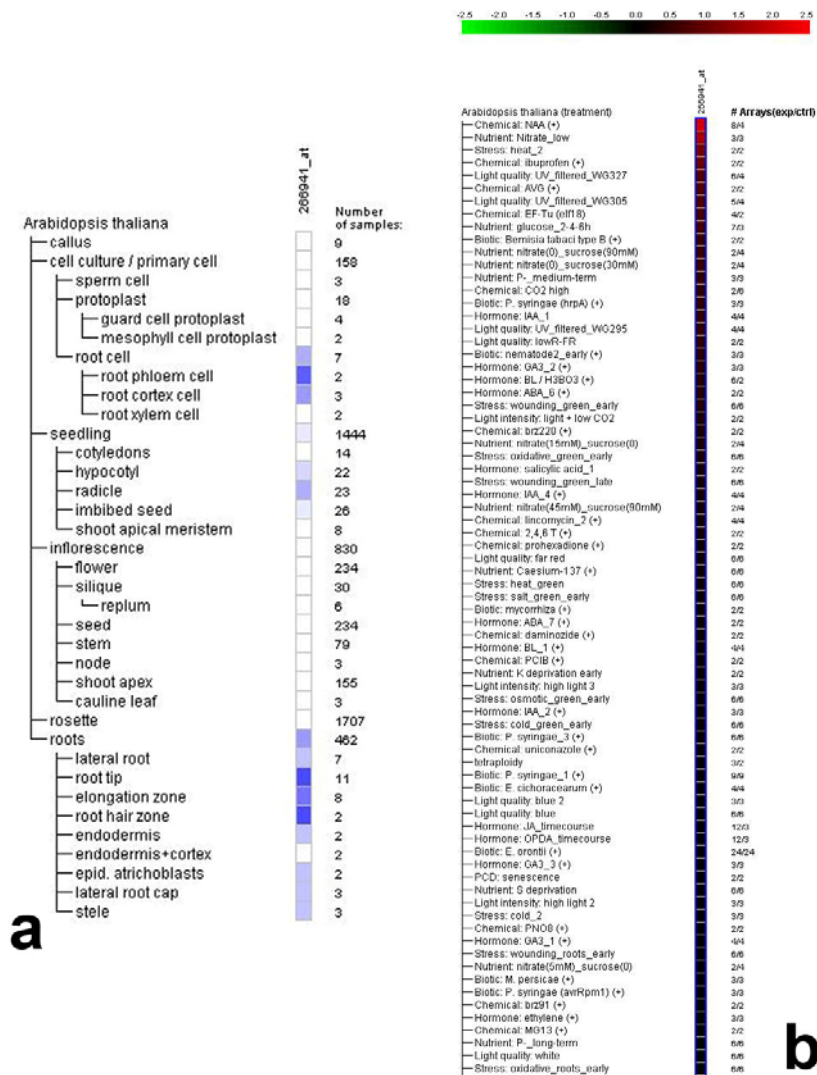


Figure 7.13 Expression of AT2G18980 in *Arabidopsis*. Gene expression based on tissue type (a) and stimulus of gene (lowest expression profiles deleted from figure for simplicity) (b). Data obtained from Genevestigator (Hruz *et al.*, 2008).

Amplification primers specific to *MecPX3* coding sequence (PX3-GSP1 and PX3-GSP2) successfully amplified a fragment approximately 1 Kb in length in a two-step/nested PCR process from the *EcoRV* digested library – the largest single product generated from the four libraries (Figures 7.12 and 7.14a). The fragment was cloned (Section 2.2.1), sequenced (Section 2.4.9) and a second pair of primers (data not shown) were designed to amplify sequence upstream. This resulted in a fragment of approximately 2.5 Kb in length from the *EcoRV* digested library (Figure 7.14b). Sequence analysis confirmed successful amplification of the region upstream of the *MecPX3* coding sequence and permitted the generation of primers to PCR-amplify an intact promoter region (approximately 2 Kb) from isolated genomic DNA (Section 2.3.1).

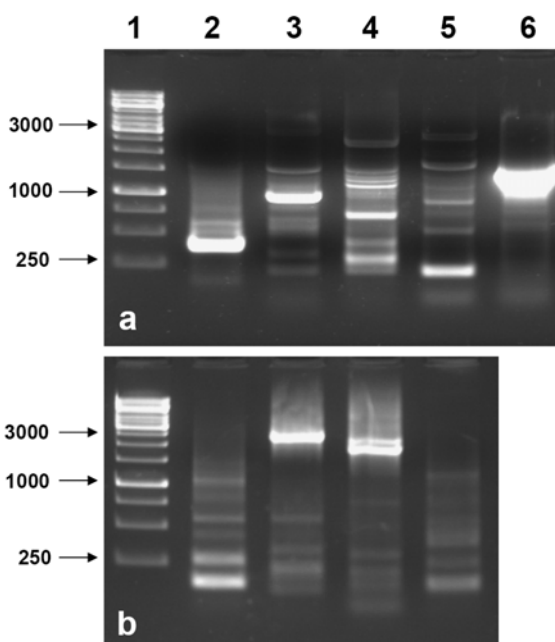


Figure 7.14 Amplification products of *MecPX3* sequence. Products following amplification with primers PX3 GSP1 (not shown) and PX3 GSP2 (**a**) and other specific primers (**b**). Template DNA from libraries digested with *DraI* (lanes 2), *EcoRV* (lanes 3), *PvuI* (lanes 4) and *StuI* (lanes 5). Positive control provided by manufacturer (lane 6). DNA ladder (bp; lanes 1). Adaptor-ligated libraries produced with the GenomeWalker™ kit (Clontech) using BRA337.

Preliminary sequence analysis of both DNA strands revealed various *cis* regulatory motifs that are involved in signal transduction, implicating *MecPX3* expression in

response to abiotic and biotic stresses. These include numerous motifs involved in light responsiveness such as the AT1-motif, I-Box and GT1-motif all of which have been characterised in *S. tuberosum*. Numerous W-Box motifs were also identified that are associated with WRKY transcription factors with roles in wound inducible expression (Hara *et al.*, 2000; Walley *et al.*, 2007). Additionally, motifs were also identified with links to phytohormones including abscisic acid, methyl jasmonate and gibberellin (Table 7.2).

Table 7.2 Regulatory motifs identified in *MecPX3* promoter sequence.

Response	Motif*	Sequence
Absciscic acid responsiveness	ABRE	ACGTGGC
	ACE	AAAACGTTTA
	AT1-motif	ATTAATTTTACA
	Box 4	ATTAAT
	Box I	TTTCAAA
Light responsiveness	G-Box	CACGTT
	GAG-motif	AGAGAGT
	GT1-motif	ATGGTGGTTGG
	I-Box	TATTATCTAGA
	TGACG-motif	TGACG
MeJA responsiveness	CGTCA-motif	CGTCA
	TATC-box	TATCCCA
Gibberellin responsiveness	P-Box	CAACAAACCCCTT
	MBS	TAACTG
Drought inducibility		

* Selection of *cis* regulatory motifs identified from PlantCARE database (Section 2.4.10).

7.4 DISCUSSION

This is the first report of cassava transformed with genes to over-express *MecSOD2* and *GalUR*, as well as their antisense forms. Importantly, transgenic cassava harbouring *GUSPlus* have been generated that should offer important insights into transgene expression driven by the *StPAT* promoter.

7.4.1 Over-expression of *GalUR* has shown to enhance ascorbate content in *planta* and is predicted to occur in pDEST™-*GalUR* cassava

The successful generation of pDEST™-*GalUR* transgenic plants is previously unreported in cassava, presenting a unique medium to study ascorbate production and its role in the crop. Over-expression of *GalUR* has been used effectively to enhance ascorbate content and improve tolerance to abiotic stress in other plant species, including *Arabidopsis* (Agius *et al.*, 2003), potato (Hemavathi *et al.*, 2009) and tomato (Oller *et al.*, 2009). It therefore seems reasonable to predict that the pDEST™-*GalUR* cassava will also have enhanced ascorbate content. Although over-expression of *GalUR* may improve tolerance to PPD, an increase in ascorbate for food/nutritional value is unlikely to have significant commercial value since cassava leaves are already relatively rich in ascorbate; in (raw) cassava roots there is approximately 14.9-50 mg 100 g⁻¹ and in leaves 60-370 mg 100 g⁻¹. In comparison, carrots have approximately 5.9 mg 100 g⁻¹ and green beans 16.3 mg 100 g⁻¹ (data cited in Montagnac *et al.*, 2009a). Whatever the levels of ascorbate in the pDEST™-*GalUR* transgenic plants, the physiological data collected here suggests the transgene does not detrimentally impact on either plant height or root stock weight. Although plant lines GalUR:BAA and GalUR:BAG were significantly taller compared with wild-type plants (based on independent-samples *t*-test), it would be prudent to await further data to determine whether this has biological significance. No phenotypic differences were observed in transgenic potato (Hemavathi *et al.*, 2009) or tomato (Oller *et al.*, 2009).

7.4.2 Assessing pDEST™-*GalUR* roots at 72 hr post-harvest improved symptom characterisation

Assessing pDEST™-*GalUR* roots at 72 hr (rather than 96 hr) post-harvest allowed improved visualisation of PPD symptoms. As discussed in Chapters 5 and 6, the roots observed at 96 hr post-harvest were often decayed and noticeably desiccated. However, the symptoms assessed at 72 hr post-harvest were more in-keeping with vascular streaking typical of PPD. Whether this is due to the shorter time period or a result of the transgene was not determined. However, wild-type root sections were also improved suggesting time was the principal cause – a logical conclusion since a further 24 hr incubation is highly likely to increase the risk of deterioration and desiccation. The distinct differentiation in symptoms between 24 hr and 72 hr time points highlights that whilst improvements have been made to the assay procedure, further work is required to

optimise the protocol to enable quantitative scoring and comparative assessments of glasshouse-cultivated roots.

7.4.3 Over-expression of pDEST™-MecSOD2 in cassava has excellent potential to modulate oxidative stress

Whilst considerable work remains to be done to fully analyse the pDEST™-MecSOD2 transformed plants, it is encouraging to note that over-expression of *SOD* in other plant species has resulted in enhanced stress tolerance. For example, over-expression of cytosolic *SOD* from tomato conferred increased tolerance to MV in sugarbeet (Tertivanidis *et al.*, 2004). If *SOD* expression is up-regulated in the transgenic cassava, it will be desirable to learn whether this alone influences the expression of genes associated with H₂O₂ breakdown. This phenomenon has been described in tobacco where over-expression of a chloroplast-targeted *Cu/ZnSOD* from pea reduced cellular damage following MV treatment but also resulted in enhanced expression of endogenous *APX* (Gupta *et al.*, 1993a; Gupta *et al.*, 1993b). Since it is assumed that H₂O₂ will accumulate, transforming plants with constructs containing *SOD* and *APX* genes would be a logical progression.

7.4.4 MecPX3 promoter is a candidate to regulate transgene expression in cassava

Isolation of the *MecPX3* promoter from genomic lambda-constructed libraries was technically challenging and unfortunately yielded very limited information. It is unknown why sequencing of the PX3ii genomic clone proved problematic but may be associated with its size, which is likely to result in primer annealing difficulties (i.e. non-specific binding) and the possibility of conformation/structural complications. There may also have been hindrances with the quantity and purity of DNA sampled, although this seems unlikely given the rigorous quality control procedures by the company undertaking the sequencing work (Lark Technologies; *pers. comm.*). The height of the problems experienced with the lambda library coincided with the transfer of work to ETH Zürich, Switzerland, to generate transgenic cassava and where alternative strategies were reviewed. The GenomeWalker™ technology proved highly effective and enabled identification and isolation of the *MecPX3* promoter region. Frustratingly, release of the cassava genome sequence (Cassava Genome Project 2009) occurred immediately after this achievement, rendering ongoing efforts to isolate the promoter somewhat redundant.

However, alignments of isolated and published sequence did enable verification of the *MecPX3* promoter region.

The various abiotic and biotic regulatory *cis* elements tentatively identified in the *MecPX3* promoter indicate suitability of the promoter for transgene expression in cassava modified to prolong shelf-life. These findings complement the expression profiles published by Reilly *et al.* (2007) and also the majority of class III peroxidases in *Arabidopsis* that are known to be expressed in roots (Tognolli *et al.*, 2002; Welinder *et al.*, 2002). Whilst the *StPAT* promoter used throughout this investigation is reportedly root specific in cassava (Ihemere *et al.*, 2006), adoption of the *MecPX3* promoter may offer a more specific role in PPD resistance.

7.4.5 Future Experiments

The achievements presented in this chapter provide a unique opportunity to gather extensive information regarding *GalUR* and *SOD* expression in cassava. Gene expression and enzyme activity data need to be obtained for selected lines of pDEST™-*GalUR* and pDEST™-*SOD* transformed plants, requiring real-time PCR experiments and the design of suitable amplification primers. Commensurate with comments in previous chapters, it seems prudent to use gene specific rather than transgene specific primers to obtain a more comprehensive view of expression *in planta*. Regarding pDEST™-*GalUR* plants, it would be interesting to measure content of ascorbate – techniques that can be performed in microplate format (Gillespie & Ainsworth, 2007; Vislisel *et al.*, 2007) or using HPLC (Epsey *et al.*, 2010; Gazdik *et al.*, 2008). The relative merits of these techniques requires review but there is the prospect to undertake HPLC work in collaboration with N. Smirnoff at the University of Exeter, UK.

Further characterisation of the *MecPX3* promoter is needed and research programmes are underway to generate deletions of the 2 Kb isolated sequence to identify minimal regions critical for expression. Given the possible effect of the *CaMV35S* promoter neighbouring the *StPAT* promoter in constructs utilised in this thesis, it may be sensible to redesign the vector to eliminate/minimise possible interference.

8 GENERAL DISCUSSION

The data presented in this thesis have advanced 25 years of cassava tissue culture research through the provision of a robust and reliable transformation protocol - an achievement that creates an essential platform for future studies. The data also reveals new and exciting information regarding the redox state of cassava *via* the measurement of thiols (glutathione and its derivatives) and highlights the crops capacity for improvement using biotechnology. Ultimately, ensuing studies should improve knowledge and provide solutions to tackle the devastating problems associated with PPD and that necessitated this research.

8.1 Optimisation of the *Agrobacterium*-mediated transformation protocol radically improved success rate

The generation of transgenic cassava has proven a nemesis for many researchers, including my former colleagues (H. Vanderschuren, *pers. comm.*; J. Owiti, *pers. comm.*; M. Stupak, *pers. comm.*; C. Faso, *pers. comm.*). The review of tissue culture techniques as well as acquisition of primary data and compilation of advice would have one believe that cassava tissue culture and transformation have mystical qualities. The results and conclusions presented in this thesis have confirmed this assertion to be utter poppycock, with the proposition that such fantastical notions are the product of political agendas. That said, *Agrobacterium*-mediated transformation of FEC is not without its complications and adoption of the original protocol(s) (Zhang & Gruissem, 2004; Zhang & Puonti-Kaerlas, 2004) seriously hampered progress of this project; almost two years research and 141 transformation attempts failed to yield transgenic plants. However, a comprehensive review of the protocol and implementation of numerous modifications ensured unrivalled success in generating transgenic material. FEC cultivation in efficiently regulated climate chambers (Sanyo MLR Plant Growth Incubator) to prevent moisture accumulation on culture dish lids and the replacement of Gelrite™ for Noble agar in media promoted consistent production of viable, non-hyperhydric FEC. Optimisation of the setting agent concentration (or type) resulted in significant improvements in tissue culture of other plant species including *P. strobus* (Garin *et al.*, 2000), *A. polyphylla* (Ivanova & Van Staden, 2011), sugarbeet (Owens & Wozniak, 1991) and interestingly Noble agar is the preferred agent used in cassava tissue culture at the DDPSC (N. Taylor, *pers. comm.*). Additionally, the abandonment of SH liquid media stages proved crucial since significant losses due to contamination (invariably 50-100%)

meant weeks of FEC cultivation were wasted and morale dwindled. Interestingly, the use of liquid media was introduced by Taylor *et al.* in (1996), who now uses only plated media (N. Taylor, *pers. comm.*). Co-cultivation of FEC and *Agrobacterium* directly on GD plates – a technique used in transformation of *B. distachyon* (Alves *et al.*, 2009), frequent use of nylon mesh to support and transfer material between media, frequent replenishment of media, optimisation of hygromycin concentrations and acclimatisation of transformed FEC collectively proved highly influential (Chapter 4) and resulted in publication of the modified *Agrobacterium*-mediated transformation protocol (Bull *et al.*, 2009).

8.2 A robust transformation system is likely to expedite cassava research

The evolution of a robust transformation protocol permitted essential progress to achieve the research objectives discussed in this thesis and also enhanced uptake of the technique in Africa. A project is currently underway - supported by the Bill & Melinda Gates Foundation - to transfer knowledge and technology to a research laboratory in Tanzania. The project was funded as a direct consequence of research presented in this thesis. Preliminary results are promising with the successful implementation of the protocol using cultivar TMS60444 and somatic embryos have also been established from farmer-preferred cultivars, including Kibandameno, TME7, Mahando, Katakya, Sagalatu, Mzungu and Milundikachini (Bull *et al.*, 2011). Similar initiatives are also being instigated in Kenya and South Africa (H. Vanderschuren, *pers. comm.*) with interest from groups in Denmark, Japan and Scotland. As cassava biotechnology is more widely adopted, we expand our understanding of the crop and should more efficiently integrate desirable traits into farmer-preferred cultivars. The continuing improvements, investment and accessibility of *Agrobacterium*-mediated transformation of FEC has in recent years led to an increase in its implementation, with publications reporting, for example, improved provitamin A content (Welsch *et al.*, 2010), protein content (Zhang *et al.*, 2003b; Abhary *et al.*, 2011), drought resistance (Zhang *et al.*, 2010), virus resistance (Vanderschuren *et al.*, 2007; Vanderschuren *et al.*, 2009; Zhang *et al.*, 2005) as well as used to study promoter characteristics (Beltrán *et al.*, 2010).

8.3 Development of cassava harbouring the *GUSPlus* reporter gene will provide insights into *StPAT* promoter expression and may influence acquisition of alternative promoters

The production of pDEST™-*GUSPlus* and pDEST™-*GUSPlus*(-)*PAT* transformed cassava will provide valuable insights into transgene expression driven by the *StPAT* promoter. As detailed in Chapter 5, transgene (*MecAPX2*) expression does not appear to be restricted to root tissue but also occurs in leaves. This result was unexpected since Ihemere *et al.* (2006) reported root-specific expression in transgenic cassava and pDEST™-*GUSPlus* is also known to be predominately expressed in *Arabidopsis* roots (Page, 2009). To what extent the neighbouring *CaMV35S* promoter may be affecting *trans* expression needs to be determined, an occurrence that has been reported previously *in planta* (Yoo *et al.*, 2005). However it is difficult to explain why this would arise in the transgenic cassava plants presented in this thesis but not in other reports. Peremarti *et al.* (2010) wrote that “currently, the creation of transgenic plants with stable gene expression relies on a healthy dose of luck...!”. These curious observations coupled with the rather mysterious origins of the *StPAT* promoter highlight the need for an extensive review and screening for potentially suitable root-specific and/or PPD-specific promoters. This was broached in this investigation with the successful isolation of the regulatory sequence of *MecPX3* (Chapter 7). Additionally, the promoter from *PtL4* linked to *GUSPlus* has been shown to confer activity predominately in transgenic cassava storage roots and vascular stem tissue (Beltrán *et al.*, 2010), whilst *pDJ3S* – the promoter controlling expression of the storage protein (dioscorin) in yam – revealed GUS activity in transgenic carrot roots (Arango *et al.*, 2010). With researchers at ETH Zürich, a comprehensive analysis of predicted root specific promoters is being initiated. Had we but world enough, and time, this new project would have been developed in this thesis.

8.4 Assessment of PPD in glasshouse-cultivated storage roots is complex but preliminary results are encouraging

The assessment of PPD in glasshouse-cultivated storage roots was undertaken using material transformed with pDEST™-*MecAPX2* (Chapter 5), pDEST™-*AtGSH1* (Chapter 6) and pDEST™-*GalUR* (Chapter 7) and which proved inconclusive. This was largely due to inappropriate time-points since most PPD ostensibly occurred between 24 hr and 96 hr post-harvest. Importantly, pDEST™-*GalUR* material was assessed at 72 hr rather than 96 hr and whilst this did improve symptom characterisation (i.e. less desiccation and general decay) it remained too complex to gather analysable data. In addition to the

protracted sampling times, symptoms observed between roots of the same line and even the same plant were surprisingly diverse. This is probably due to root size and stage of development (Wheatley *et al.*, 1985). Difficulty characterising storage roots is not confined to the glasshouse-cultivated material but also field roots; Salcedo *et al.* (2010) showed there was no correlation between the accumulation of hydroxycoumarins (measuring fluorescence using image analysis software) and visual assessment, suggesting that whilst visual assessment may be susceptible to personal interpretation it is apparently the preferred approach. One possible solution to some of the problems encountered could be to use the “whole root method” on only the largest root of each plant. It would appear advantageous to also use an earlier “late” time point, for example 48 hr rather than 72 hr or 96 hr, in an attempt to gain greater differentiation between lines. It is apparent that further research is required to optimise a suitable protocol. Ultimately, it would be desirable to establish the transgenic plants in confined field trials to measure the true impact of the transgenes on PPD in mature plants - a goal that may be possible with collaborators in South America (J. Beeching, *pers. comm.*).

8.5 Extensive collection of transgenic cassava is a valuable tool to assess ROS modulation and antioxidant status

More than 150 characterised *in vitro* cassava plantlets transformed with pDEST™-*MecAPX2*, pDEST™-*AtGSH1*, pDEST™-*MecSOD2* and pDEST™-*GalUR*, comprising more than 40 independent lines, have been generated as part of this investigation. This constitutes the largest worldwide collection of cassava with transgenes implicated in modulating oxidative stress. As discussed in Chapters 5-7, the vast majority (>85%) of the lines comprised a single genomic fragment containing T-DNA, whereas only 30-40% has been reported in other publications (Ihemere *et al.*, 2006; Zhang *et al.*, 2003b). Why this phenomena should occur is unknown and transgene copy number remains to be determined, but certainly it is promising for developing cassava for commercialisation. Preliminary analyses of the transgenic plants also revealed stable integration of the transgenes and thus serve as an important tool to assess ROS modulation during PPD. As discussed, analyses remain within the early phase and numerous experiments need to be completed to fully characterise the plants. Notwithstanding, available data is very encouraging with over-accumulation of transgene transcripts and enzyme activity in pDEST™-*MecAPX2* plants and the chance that pDEST™-*GalUR* plants have enhanced ascorbate content. The confirmed accumulation of glutathione in pDEST™-*AtGSH1* cassava based on HPLC analysis, as well as ratios between GSH and GSSG in roots

and leaves is an interesting result and requires further investigation to establish the homeostasis between GSH, GSSG and cysteine flux (Chapter 6). Whether or not the various transgenic plants have improved resistance to ROS remains to be identified, but it is reassuring that transgenic plants are morphologically commensurate (based on plant height and root stock weight) with wild-type plants. This provides confidence that the modified expression cassette (Chapter 3) seemingly does not detrimentally affect transformed plants. Lastly, the transformation of FEC with single transgenes is providing valuable information regarding their role and affect on PPD. However, there is now scope to combine transgenes in multigene constructs in an attempt to expedite ROS modulation. This is particularly relevant in plants transformed with pDEST™-MecSOD2 where increased activity may result in enhanced levels of H₂O₂. Chloroplast-targeted *Cu/ZnSOD* and *APX* have been used to transform sweet potato effecting enhanced drought tolerance. Interestingly, tuber size was detrimentally affected but this was attributed to an insufficiency of water for development (Lu *et al.*, 2010). Similarly, a cytosolic *Cu/ZnSOD* and *APX* combination in transgenic tobacco improved H₂O₂ levels compared with wild-type material (Faize *et al.*, 2011).

The achievements detailed in this investigation have consigned problems associated with *Agrobacterium*-mediated cassava transformation to the history books. In-turn this enabled the generation of a plethora of transgenic cassava harbouring genes potentially able to minimise the devastating effects of PPD. The unique transgenic plants serve as an exceptional platform for future research and will allow rapid expansion of our knowledge of this staple food crop.

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